약학박사 학위논문

Identification of YH18421, a novel GPR119 agonist for the treatment of type 2 diabetes

제 2 형 당뇨병 치료를 위한 새로운 GPR119 작용제로써 YH18421 동정

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ABSTRACT

Identification of YH18421, a novel GPR119 agonist for the treatment of type 2 diabetes

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G-protein-coupled receptor 119 (GPR119) represents a promising target for the treatment of type 2 diabetes as it can increase both GLP-1 secretion from intestinal L cells and glucose-stimulated insulin secretion (GSIS) from pancreatic cells. Due to these dual mechanism of action, the development of small molecule GPR119 agonists have received much interest for the treatment of type 2 diabetes. Here, YH18421, a novel small-molecule GPR119 agonist was identified and its therapeutic potential was evaluated.

YH18421 specifically activated human GPR119 with high potency and potentiated GLP-1 secretion and GSIS *in vitro* cell based systems. In normal mice, single oral administration of YH18421 improved glucose tolerance. Combined treatment of YH18421 and the DPP-IV inhibitor augmented both plasma active GLP-1 levels and glycemic control. In diet induced obese (DIO) mice model, glucose lowering effect of YH18421 was maintained after 4 weeks of repeat dosing and YH18421 acted additively with DPP-IV inhibitor. YH18421 also inhibited weight gain during 4 weeks of administration in DIO mice. These results demonstrate that YH18421 is capable of delivering sustained glucose control and preventing weight gain and suggest that YH18421 alone or in combination with a DPP-IV inhibitor, represents a new type of an oral GLP-1 based therapy for the treatment of type 2 diabetes.

Keywords **: YH18421/ GPR119/ DPP-IV inhibitor/ GLP-1/ Insulin/ Type 2 diabetes**

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AMPK	Adenosine monophosphate activated protein kinase
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CPT	Carnitine palmitoyl transferase
CRE	cAMP response element
DMEM	Dulbecco's modified eagle medium
DMPK	Drug metabolism and pharmacokinetics
DMSO	Dimethylsulfoxide
DPP-IV	Dipeptidylpeptidase-IV
EC_{50}	Half maximal effective concentration
EMEM	Eagle's Minimum essential medium
ELISA	Enzyme linked immunosorbent assay
FFA	Free fatty acid
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GPCR	G protein-coupled receptor
GPR119	G protein coupled receptor 119
GLP-1	Glucagon like peptide-1
GSIS	Glucose stimulated insulin secretion
HbA1c	Glycated haemoglobin A1c
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGP	Hepatic glucose output
HDL	High density lipoprotein
HTS	High throughput screening
LDL	Low density lipoprotein
MC	Methyl celluose
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
OEA	Oleylethaolamide
PPAR	Peroxisome proliferator-activated receptor
PGC	PPAR gamma coactivator

PYY	Peptide YY
PXR	Pregnane X receptor
PXRE	PXR response element
qPCR	Quantitative polymerase chain reaction
RXR	Retinoid X receptor
SEM	Standard error of the mean
SGLT	Sodium/glucose co-transporter
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

INTRODUCTION

I. Type 2 diabetes mellitus (T2DM)

Diabetes mellitus is a heterogeneous group of disorders characterized by high blood glucose levels. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the

hyperglycemia (American Diabetes Association 2001)

There are two main types of diabetes, type 1 and type 2 with their clinical relevance shown in Table 1. Type 1 or insulin-dependent diabetes mellitus results from an absolute deficiency of insulin due to autoimmunological destruction of the insulin-producing pancreatic β cell (Atkinson et al. 2001). In type 2 or non-insulin-dependent diabetes mellitus, muscle and fat cells are 'resistant' to the actions of insulin and compensatory mechanisms that are activated in the β cell to secrete more insulin are not sufficient to maintain blood glucose levels within a normal physiological range (Cavaghan et al. 2000; Kahn 2001). Type 2 diabetes (T2DM) is far more common (accounting for more than 90% of all diabetes cases) than type 1 diabetes mellitus (T1DM).

T2DM pathophysiology involves defects of metabolism in at least seven major organs and tissues, including the pancreas, liver, skeletal muscle, adipose tissue, brain, gastrointestinal tract, and kidney (Figure 1). Reduced sensitivity to insulin (i.e., impaired insulin-mediated glucose disposal or insulin resistance) in liver, muscle and adipose tissue, progressive decline in pancreatic β cell function leading to impaired insulin secretion, loss of satiety in brain, increased glucose

reabsorption in kidney, and decreased GLP-1 release in intestine eventually result in hyperglycemia, the hallmark feature of T2DM (DeFronze 2009; Cornell 2015).

T2DM is a complex chronic disorder that requires continuous medical care, management for control of abnormal glucose levels, and multifactorial risk reduction strategies to normalize blood glucose levels to prevent or minimize acute and long-term microvascular complications (including retinopathy, nephropathy, and neuropathy), macrovascular complications (such as a heart attack and stroke) and the need for lower limb amputations (Taylor et al. 1994; DeFronzo et al. 1999).

T2DM has become a major global public health concern that affects an estimated 8.3% of the adult population or 382 million people worldwide. If current trends continue, it is estimated that 592 million people worldwide (~ 10% of the adult population) will have T2DM by 2035 (International Diabetes Federation 2013).

Table 1. Types of diabetes mellitus with clinical relevances

	T1DM	T2DM
Clinical	Onset < 20 years Normal weight Decreased blood insulin Anti-islet cell antibodies Ketoacidosis common	Onset > 30 years Obesity Normal or increased blood insulin No anti-Islet cell antibodies Ketoacidosis rare
Pathogenesis	Auto-immunity Severe insulin deficiency	Insulin resistance Relative insulin deficiency
Islet cell	Early insulitis Marked atrophy and fibrosis Severe β cell depletion	No early insulitis Focal atrophy and amyloid deposits Mild β cell depletion
Prevalence	< 10%	> 90%

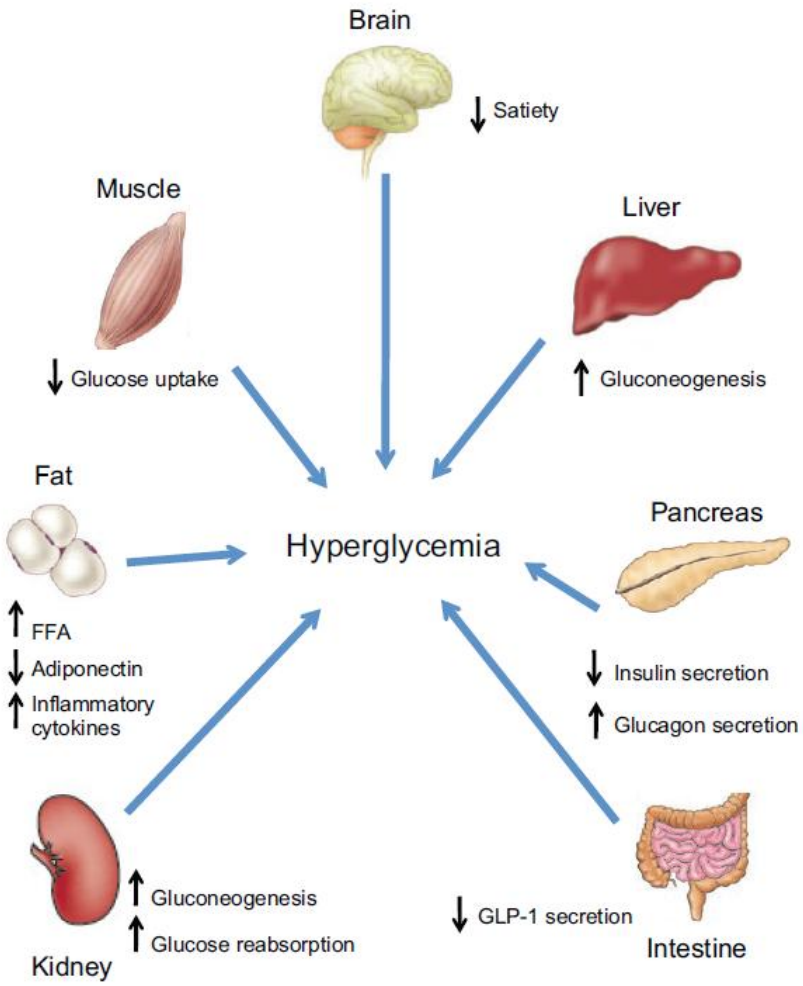


Figure 1. Pathophysiology of type 2 diabetes (Cornell 2015).

II. Pharmacological treatments for T2DM

Several pharmacological treatments aimed toward glycemic control are currently available, either as monotherapies or combination therapies, each with its own limitations and potential risks (Goldberg et al. 2008; DeFronzo et al. 2015). These can be classified based on their mechanisms of action and structural features (Table 2).

1. Metformin

Metformin is the most widely prescribed antidiabetic medication worldwide and works by suppressing hepatic glucose production, leading to a reduction in fasting plasma glucose levels and glycated haemoglobin A1c (HbA1c) (Cusi et al. 1996). The mechanisms by which metformin suppresses hepatic glucose production remain unclear but include inhibition of mitochondrial complex I, activation of AMP-activated protein kinase (AMPK), and inhibition of gluconeogenic enzymes (Ferrannini 2104; Madiraju et al. 2014). Although metformin is generally well tolerated, drug-induced lactic acidosis can be a potentially fatal adverse effect, and several contraindications to the use of this agent have been suggested (Bailey 2008).

Table 2. Characteristics of major classes of anti-diabetic agents

Drugs	Efficacy	Mechanisms	Body weight	Side effects
Metformin	↓↓	↓HGP	↓	Gastrointestinal Lactic acidosis
Sulfonylurea	↓↓	↑Insulin	↑	Hypoglycemia
Thiazolidinedione	↓↓	↑Insulin sensitivity	↑	Edema Bone loss
SGLT-2 inhibitor	↓↓	↑Glucosuria	↓	Genital infection
GLP-1 agonist	↓↓	↑Insulin ↓Glucagon	↓	Gastrointestinal
DPP-IV inhibtor	↓	↑Insulin ↓Glucagon	Neutral	
Insulin	↓↓↓	↓HGP ↑Glucose uptake	↑	Hypoglycemia

* HGP, hepatic glucose production

2. Sulfonylureas

Sulfonylureas augment insulin secretion from pancreatic β cells, and the resulting hyperinsulinaemia overcomes insulin resistance. These agents act in a glucose-independent manner, and allow insulin secretion to occur even if the glucose level is low, resulting in hypoglycemia (Bodmer et al. 2008). Also, after the initial decline, HbA1c rises progressively because sulfonylureas have no long-term protective effect on β cell function and might even accelerate failure of β cell function. Sulfonylureas commonly are associated with weight gain, and some retrospective studies suggest that they might increase cardiovascular events (Turner et al. 1999; Simpson et al. 2006).

3. Thiazolidinediones

Thiazolidinediones are the only currently available insulin-sensitizing agents. They enhance insulin action in skeletal and cardiac muscle, the liver and adipocytes by activation of PPARγ. Multiple mechanisms mediate their insulin-sensitizing effects; increased insulin signaling, stimulation of several intracellular steps involved in glucose uptake, stimulation of fat oxidation, proliferation of subcutaneous adipocytes and activation of genes involved in lipogenesis, fat

redistribution from visceral to subcutaneous stores, reduced plasma levels of FFAs, a reduction in circulating inflammatory cytokines, and an increase in adiponectin levels (Eldor et al. 2013). Unfortunately, thiazolidinediones have side-effects associated with edema, weight gain, an increased risk of bone fracture and heart failure, thus are contraindicated in patients at high risk for congestive heart failure (Nesto et al. 2003).

4. Sodium/glucose co-transporter 2 (SGLT2) inhibitors

SGLT2 inhibitors block glucose absorption in the proximal renal tubule. They decrease the maximum renal glucose reabsorptive capacity and, importantly, reduce the blood glucose by spilling glucose into the urine. The increased glucose removal from the body via glucosuria leads to a reduction in plasma glucose, however, given their mode of action, SGLT2 inhibitors are less effective in lowering blood glucose in patients with impaired renal function. Adverse effects include genital mycotic infections in female patients, balanitis in uncircumcized male patients, urinary tract infections and volume-related side effects in older patients and individuals taking diuretics (Abdul-Ghani et al. 2011).

5. Glucagon like peptide-1 (GLP-1) modulators

Incretins are a group of metabolic hormones released after eating and stimulate a decrease in blood glucose levels. The two main molecules that fulfill criteria for an incretin are the intestinal peptides glucagon like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). The incretin hormone GLP-1 is synthesized in and secreted from enteroendocrine L cells found throughout the small and large intestine, after posttranslational processing of proglucagon by prohormone convertase 1. GLP-1 stimulates glucose-dependent insulin secretion and suppresses glucagon secretion, gastric emptying and appetite. In addition, activation of GLP-1 enhances β-cell proliferation in rodents and inhibits β-cell apoptosis in rodent and human islets. Besides its role in glucose metabolism, activation of GLP-1 receptors by GLP-1 receptor agonist also leads to pleiotropic non-glycemic effects, which are metabolically favorable, in multiple tissues through direct actions on tissues expressing GLP-1 receptors (Figure 2) (Campbell and Drucker 2103). Although these characteristics of GLP-1 make it strong potential as chronic therapies for diabetes, GLP-1 is subject to rapid amino-terminal degradation ($t1/2 \sim 1$ min) by dipeptidyl peptidase–IV (DPP-IV), a proline-specific serine dipeptidase. GLP-1 thus becomes

inactivated by DPP-IV to generate GLP-1 [9–36] (Figure 3) (Moller 2001). This issue has been addressed with the discovery of DPP-IV-resistant GLP-1 receptor agonists such as exenatide, liraglutide, and dulaglutide, which exhibit glucoregulatory activities similar to natural GLP-1. Nausea and vomiting are the most common side effects with GLP-1 receptor agonists, In addition to gastrointestinal side effects, a disadvantage of GLP-1 receptor agonists is that it requires parenteral administration (Trujillo et al. 2015).

Another strategy to enhance GLP-1 function consists of blocking endogenous GLP-1 inactivation through the selective inhibition of DPP-IV. DPP-IV inhibitors prolong the half-life of endogenously secreted GLP-1. Orally active DPP-IV inhibitors such as sitagliptin and linagliptin have been approved for clinical use. As DPP-IV inhibitors do not increase (only prolong) plasma GLP-1 levels, their ability to augment insulin secretion and reduce HbA1c is modest (Aroda et al. 2012) and they do not elicit the weight loss observed following treatment with GLP-1 receptor agonists (Drucker and Nauck 2006; Brubaker 2007). Generally, DPP4 inhibitors have a favorable safety profile with no serious side effects (Drucker 2013).

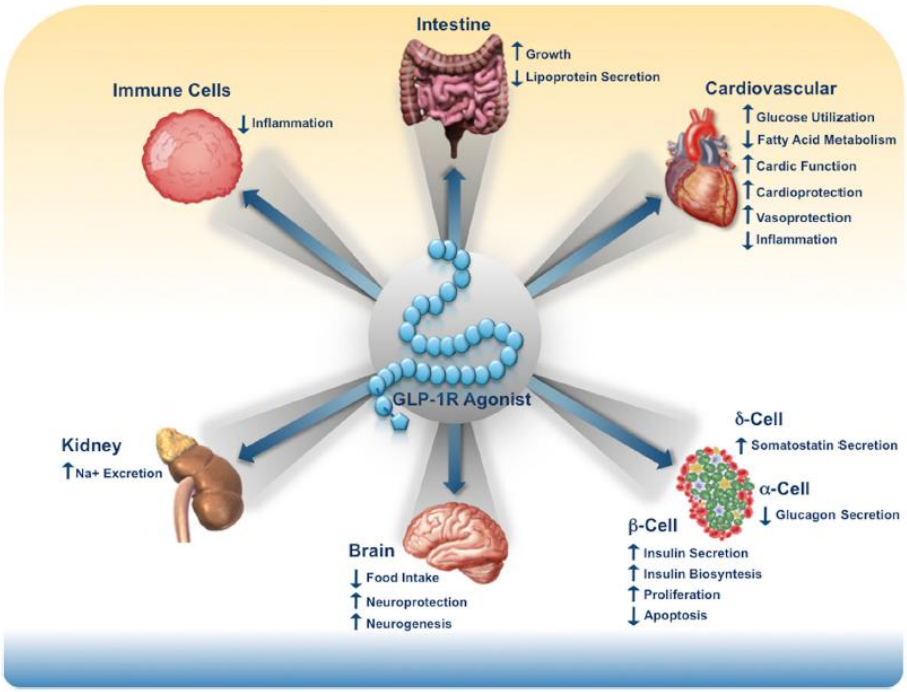


Figure 2. Pharmacological actions of GLP-1 agonist (Campbell and Drucker 2103).

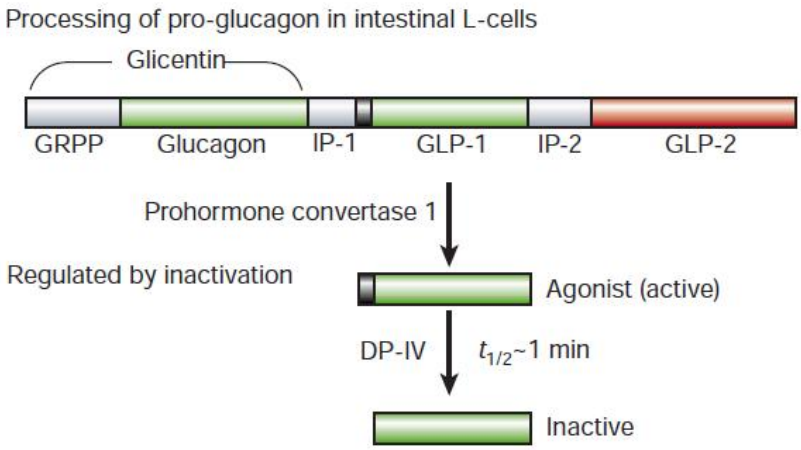


Figure 3. Biosynthesis and regulation of GLP-1. Pro-glucagon is cleaved by prohormone convertase 1 to generate active GLP-1[7-36], which is released from intestinal L-cells during nutrient ingestion. GLP-1 is rapidly hydrolysed by DPP-IV to produce an inactive product, GLP-1[9-36] (Moller 2001).

6. Insulin

If oral or injectable antidiabetic agents fail to normalize HbA1c, patients with T2DM can be treated with insulin and insulin analogues. In new-onset T2DM, intensive insulin therapy to reverse the metabolic decompensation, such as glucotoxicity and lipotoxicity, has proved effective in maintaining glycaemic control (HbA1c ~ 6.0 %) for long periods. Combining insulin therapy with oral antidiabetic agents (metformin, sulfonylurea, thiazolidinedione, DPP-IV inhibitor, SGLT-2 inhibitor) or GLP-1 receptor agonists can effectively improve glycaemic control and enable insulin dose reduction (Hu et al. 2011). However, safety concerns regarding hypoglycemia and weight gain, and the practical barrier of drug administration by injection have limited the use of this therapy (Holman et al. 2007). The two main types of insulin analogues are bolus or rapid-acting analogues, which are used for mealtime glucose control and pump administration, and basal or sustained-action analogues, which are used for daytime and night time glucose control. A basal insulin analogue is intended to mimic the steady, unprovoked secretion profile of a healthy pancreas (Owens et al. 2014; Zaykov et al. 2016).

III. GPCR in energy metabolism

G protein-coupled receptors (GPCRs) constitute by far the largest receptor family in mammals and are involved in the regulation of virtually all cellular and physiological functions in the body. These receptors typically couple to specific G protein families, such as Gαs, Gαi/o, Gαq/11, and others. Coupling to Gαs stimulates adenylyl cyclase, which increases intracellular cyclic AMP levels. Coupling through Gαi/o inhibits adenylyl cyclase, decreases intracellular cyclic AMP levels and stimulates mitogen-activated protein kinase (MAPK). Gαq/11 activation causes stimulation of phospholipase C (PLC) enzymes, which hydrolyse membrane phospholipids to release inositol 1,4,5-triphosphate (IP3) and diacylglycerols (DAGs). The release of these signaling molecules leads to increased intracellular calcium concentrations and stimulation of protein kinase C (PKC). (Oh and Olefsky 2016).

GPCRs have traditionally been regarded as receptors for hormones, neurotransmitters and other mediators, which are produced solely for the purpose of carrying a signal and to serve cell–cell communication. This view is now changing as a growing number of

GPCRs are being identified for which the ligands are energy substrates (such as fatty acids and glucose) or metabolic intermediates (such as acetate, lactate or ketone bodies). By activating specific cognate GPCRs, carbohydrate or lipid metabolites can act in a hormone-like manner in addition to functioning as sources of energy. The regulation of energy metabolism requires the well-balanced control of opposing metabolic pathways such as lipolysis and lipogenesis, glycolysis and gluconeogenesis, or fatty acid oxidation and synthesis which is often disturbed in metabolic diseases such as obesity, type 2 diabetes and dyslipidaemia. Given their central role in the coordination of metabolic processes, metabolite-sensing GPCRs are possible targets for anti-diabetic drugs (Blad et al. 2012).

GPCRs, including GPR119, expressed by GLP-1 producing enteroendocrine L cells or insulin producing pancreatic β cells are of particular interest and have emerged as potential drug targets for the development of anti-diabetic therapeutics (Figure 4, 5) (Ahrén 2009; Reimann et al. 2012).

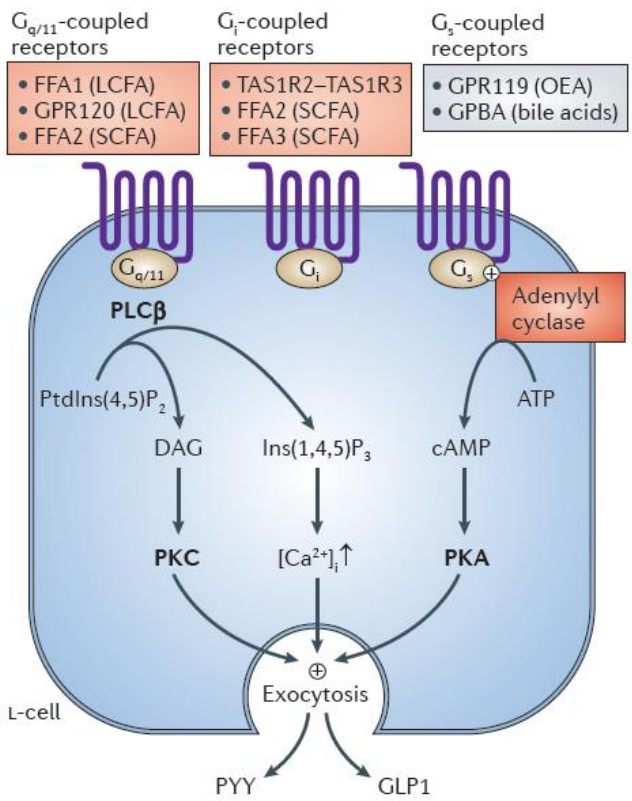


Figure 4. GPCRs involved in GLP-1 secretion in intestinal L cells. FFA, free fatty acid; Ins(1,4,5)P3, inositol-1,4,5-trisphosphate; LCFA, long-chain fatty acid; PKA, protein kinase A; PLCβ, phospholipase Cβ; PtdIns(4,5)2, phosphatidyl inositol-4,5-bisphosphate; SCFA, short-chain fatty acid; TAS1R2, taste receptor type 1 member 2 (Blad et al. 2012).

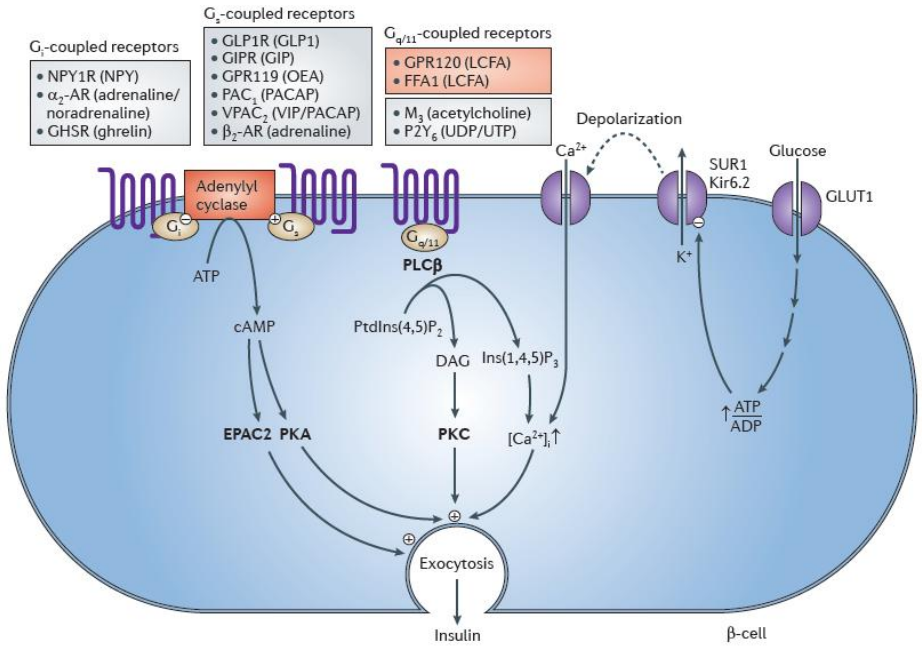


Figure 5. GPCRs involved in insulin secretion in pancreatic β cells.

α2-AR, α2-adrenergic receptor; β2-AR, β2-adrenergic receptor; EPAC2, exchange protein directly activated by cAMP2; GHSR, growth hormone secretagogue receptor; GIP, gastric inhibitory peptide; GIPR, GIP receptor; M3, muscarinic acetylcholine receptor M3; NPY, neuropeptide Y; NPY1R, NPY receptor 1; P2Y6, P2Y purinergic receptor 6; PACAP, pituitary adenylyl cyclase-activating polypeptide; PAC1, PACAP receptor 1; PtdIns(4,5)P2, phosphatidyl inositol-4,5-bisphosphate; VIP, vasoactive intestinal peptide; VPAC2, VIP receptor 2 (Blad et al. 2012).

IV. GPR119

1. Structure

GPR119 is a member of family A, rhodopsin-like GPCRs, with seven transmembrane domains connected by alternating extracellular loops and intracellular loops. The gene for GPR119 is located on the short arm of X-chromosome (Xp26.1) containing a single exon (ENST00000276218) with no introns. GPR119 is encoded by a single transcript with an open reading frame of 1008 base pairs producing a protein of 335 amino acid residues (Figure 6) (Mo et al. 2014).

GPR119 is unusual in that it does not share close primary sequence homology with other cell surface receptors encoded within the human genome and its closest homologs are believed to be the adenosine (A1 and A3) and cannabinoid receptors, which share 28% sequence identity with GPR119 in the transmembrane regions (Costanzi et al. 2008). The amino acid sequence of GPR119 is highly conserved across several mammalian species including the mouse, rat, dog, and monkey. It has been noted that the rat receptor shares 96% amino acid identity with the murine sequence, while human and mouse share 82% sequence identity (Swaminath 2008).

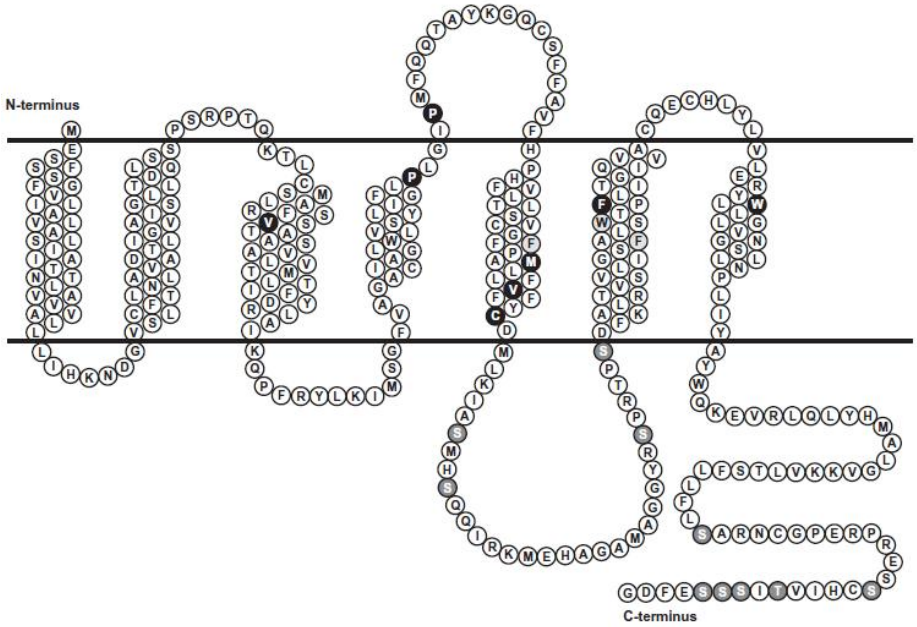


Figure 6. Human GPR119 membrane topology. The 10 amino acid residues that were predicted to be phosphorylated are indicated in white letter and shaded in dark gray. The three amino acid residues that were identified important for receptor basal activity are indicated in black letter and shaded in light gray. The eight amino acid residues that were predicted forming binding pocket for a synthetic agonist are indicated in white letter and shaded in black (Mo et al. 2014).

2. Tissue distribution

The expression of GPR119 is highly restricted in humans: the pancreas, fetal liver and gastrointestinal tract have been identified as the major sites of expression. In rodents, however, GPR119 is expressed in many regions of the brain in addition to the pancreas and gastrointestinal tract (Figure 7) (Chu et al. 2008). Within the pancreas, GPR119 expression is greatly enriched in the islet cells in both humans and rodents. An *in situ* hybridization study of rat pancreatic sections suggested that GPR119 is expressed in the β cell population. High expression levels in pancreatic β cell lines NIT-1, MIN6 and HIT-T15 provided further evidence that the β cells are the major site of GPR119 expression within the pancreatic islets (Soga et al. 2005; Chu et al. 2008). In addition to the pancreas, significant expression of GPR119 has been detected in regions of the human and rodent gastrointestinal tract. GPR119 is present in several rodent GLP-1 secreting L-cell lines including STC-1, GLUTag, FRIC and hNCI-H716 cells, as well as in mouse L-cell primary cultures (Chu 2008; Lauffer 2009). The distribution of GPR119 in subregions of the human gastrointestinal tract (duodenum, jejunum, ileum, stomach and colon) has also been reported (Chu et al. 2008).

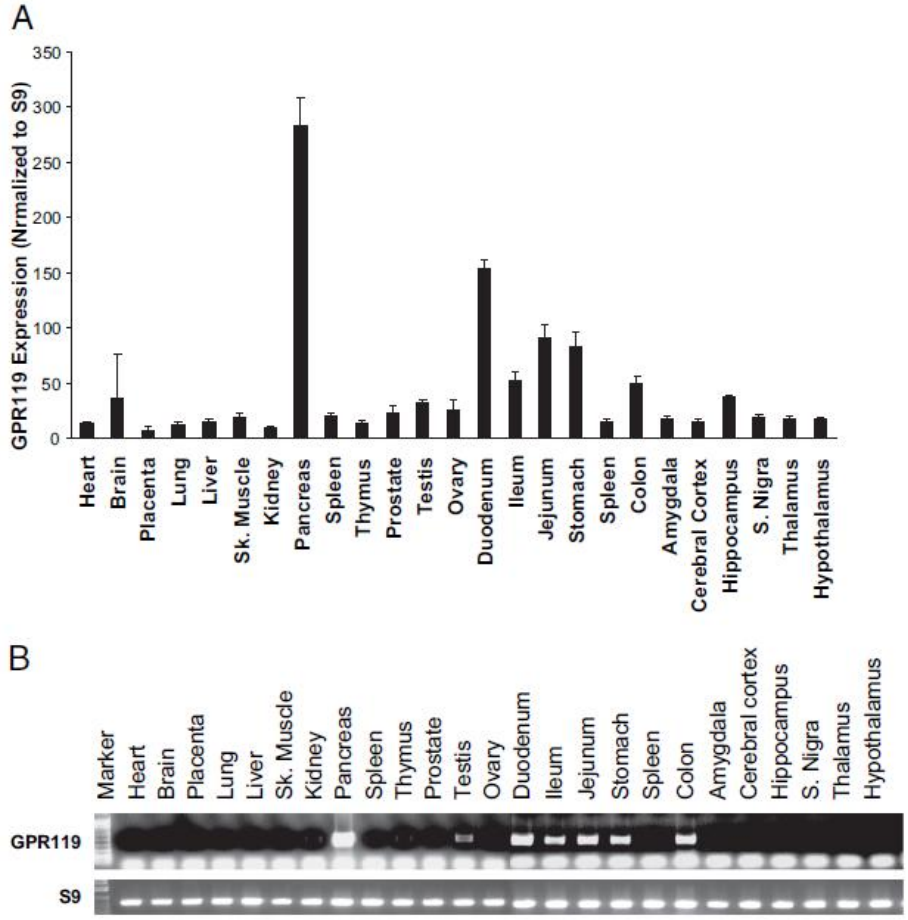


Figure 7. Tissue distribution of human GPR119. A, Taqman analysis of human GPR119 expression. B, RT-PCR analysis of human GPR119 expression (Chu et al. 2008).

3. GPR119 signaling and de-orphanization

GPR119 is coupled predominantly to the signal transducer Gαs, and that activation of the receptor results in increased adenylate cyclase activity and a rise in intracellular cAMP. Several groups have substantiated this conclusion by showing that agonists of GPR119 increase cAMP in cells that have been either stably or transiently transfected with cDNA encoding GPR119, whereas an increase in cAMP is not seen in untransfected cells (Lan 2009; Chu et al. 2010). In support of this, activation of GPR119 leads to downstream activation of the cAMP target, protein kinase A (PKA), since the purportedly selective inhibitor of PKA, H89, was an effective antagonist of GPR119 responses in intestinal cells (Lauffer et al. 2009). The poor coupling efficiency of GPR119 to Gαi and Gαq suggests that this receptor selectively couples to Gαs (Chu et al. 2007).

Initially, GPR119 was classified as an orphan receptor but in 2005 lysophospholipids containing oleic acid, palmitic acid or stearic acid were found to have agonist activity and the anorectic lipid oleoylethanolamide (OEA) was also shown to be a potent and efficacious GPR119 agonist in 2006. Later, several other endogenous lipid metabolites were shown to be GPR119 agonists in transfected cell

lines (Soga et al. 2005; Overton et al. 2006) (Figure 8). The identification of OEA as a potential endogenous ligand for GPR119 was of particular interest, since this compound has been reported to produce a number of pharmacological effects in rodent studies including reducing food intake and body weight gain. (Overton 2008). The endovanilloid compounds oleoyl dopamine (OLDA) have recently been described as GPR119 agonist with *in vitro* potencies similar to that of OEA. Moreover, *in vivo* studies demonstrated that oral administration of OLDA improved oral glucose tolerance in mice; these effects were absent or attenuated in GPR119 null mice (Dhayal and Morgan 2010; Zhu et al. 2013). These fatty acid amides, OEA and OLDA, represent the best candidates for endogenous ligands, although they are less potent and selective than the natural ligands identified for many other GPCRs. Nonetheless, this study raises the possibility that other lipid amides may play a physiological role via GPR119 signaling (Overton et al. 2006; Dhayal and Morgan 2010).

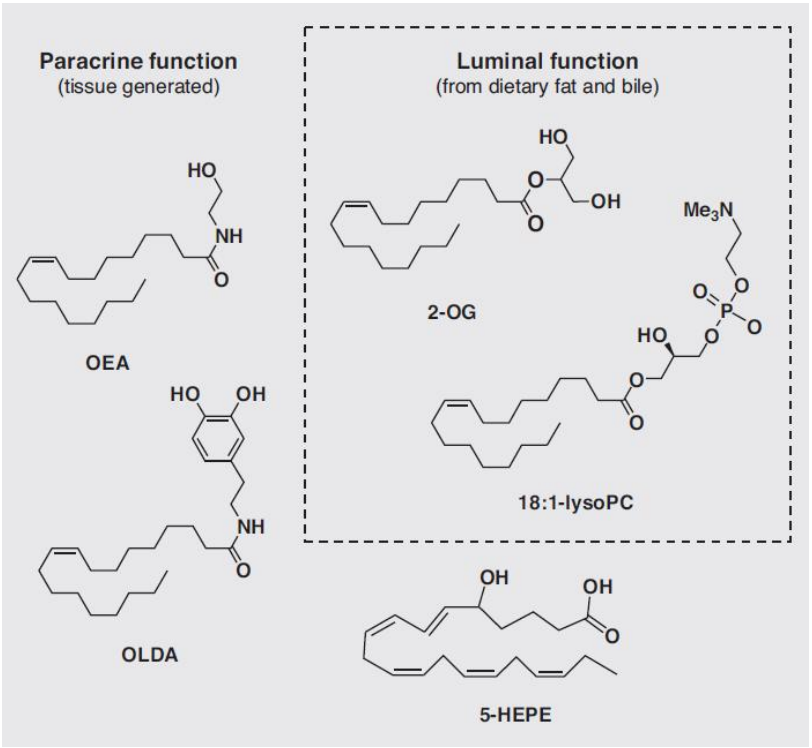


Figure 8. Major lipid metabolites proposed to be endogenous GPR119 ligands. OEA, Oleoylethanolamide; OLDA, N-oleoyl-dopamine; 18:1-lysoPC, 1-oleoyl-lysophosphatidylcholine; 2-OG, 2-oleoyl glycerol; 5-HEPE, 5-hydroxyeicosapentaenoic acid (Hansen et al. 2012).

4. Physiological functions of GPR119

4-1. Glucose stimulated insulin secretion (GSIS)

GPR119 has been identified as an insulinotropic receptor since it is expressed by β cells and the binding of agonists leads to an increase insulin release via a rise in cAMP production. Although the mechanism by which insulin secretion is increased following the activation of GPR119 involves a rise in cAMP, potentiation of insulin secretion is also dependent on the closure of ATP-sensitive K^+channels and the consequent gating of voltage sensitive calcium channels (Ning et al. 2008). The potent, selective GPR119 agonist discovered at Arena Pharmaceuticals, Inc., AR231453, significantly increased insulin release in HIT-T15 cells (a hamster insulinoma-derived cell line with robust GPR119 expression) and in rodent islets. By contrast, no effect of this compound could be seen in islets isolated from GPR119-deficient mice, confirming that its effects were indeed mediated by GPR119. Moreover, oral administration of AR231453 significantly improves glucose tolerance in a dose-dependent manner in both normal and diabetic mice and rat models, most likely due to enhanced insulin secretion through activation of GPR119. Indeed, the effect of AR231453 on glucose homeostasis is abolished in GPR119 knockout

mice (Chu et al. 2007).

4-2. Gastrointestinal hormone secretion

GPR119 improves glucose homeostasis through not only stimulating the glucose-dependent insulin secretion but also stimulating the release of incretin hormones, such as GLP-1 and GIP. Incretins are gut hormones that are secreted from enteroendocrine cells into the blood within minutes after food ingestion. The effectiveness of AR231453 on regulating glucose homeostasis is reduced by almost 50% when glucose is administered intraperitoneally, suggesting that modulation of incretin-based mechanism might also be involved in GPR119 actions (Chu et al. 2007). AR231453 stimulated GLP-1 release both *in vitro* in GLUTag cells and *in vivo* in C57BL/6 mice. In addition to increasing GLP-1 levels, AR-231453 also enhanced GPR119-mediated release of GIP from K cells *in vivo* (Chu et al. 2008).

Peptide YY (PYY), the L-cell-derived hormone, is predominantly expressed in the ileum and the colon (Sundler et al. 1993), and circulating PYY is known to reduce food intake after feeding to exert a beneficial hypophagic action (Chan et al. 2006; Field et al. 2010). GPR119 agonism has been reported to increase circulating PYY levels

in animal models as well as healthy humans (Katz et al. 2011; Ohishi and Yoshida 2012). Activation of GPR119 by AR231453 results in significantly increased plasma levels of PYY *in vivo* (Cox et al. 2010; Flock et al. 2011).

4-3. Gastric emptying and hypophagic effect

Increased release of GLP-1 and PYY which follows from GPR119 activation in intestinal L cells may be responsible for the reduction in food intake and consequent weight loss, because GLP-1 and PYY increase satiety and modulate gastric emptying (Overton et al. 2008). In support of this, selective GPR119 agonist PSN-632408 reduces food intake both acutely and during more chronic treatment, in either obese or normal male rats (Overton et al. 2006). Also, GPR119 activation with AR231453 leads to significantly delayed gastric emptying in wild type mice. This inhibitory actions of AR231453 on gastric emptying are indeed GPR119-specific, since the inhibitory effect was abolished in GPR119 KO mice (Flock et al. 2011).

4-4. β cell replication and protection

Several GPR119 agonists, including endogenous ligand OEA and

synthetic ligands PSN-632408 and AR23145351, stimulate β cell replication both *in vitro* in cultured mouse islets and *in vivo* in mouse islet grafts. In diabetic mice with islet transplantation, GPR119 agonist-treated diabetic recipient mice achieved significantly earlier normoglycemia than vehicle treated mice, thus suggesting that GPR119 agonists are potentially useful in improving islet graft function. (Gao et al. 2011). The proliferative effect of GPR119 was further supported by other studies. After 3 weeks of treatment with GPR119 agonist AS1535907 in *db/db* mice, the number of insulin- and proliferation cell nuclear antigen-positive cells and islet area is significantly higher than those in the vehicle-treated mice, suggesting the beneficial effects of GPR119 agonist on β cell proliferation and protection (Yoshida et al. 2011). Also, 7-week treatment with GPR119 agonist PSN-632408 alone or combined with DPP-IV inhibitor sitagliptin significantly stimulates β cell proliferation and increases β cell mass in diabetic C57BL/6 mice induced by streptozotocin (Ansarullah et al. 2013).

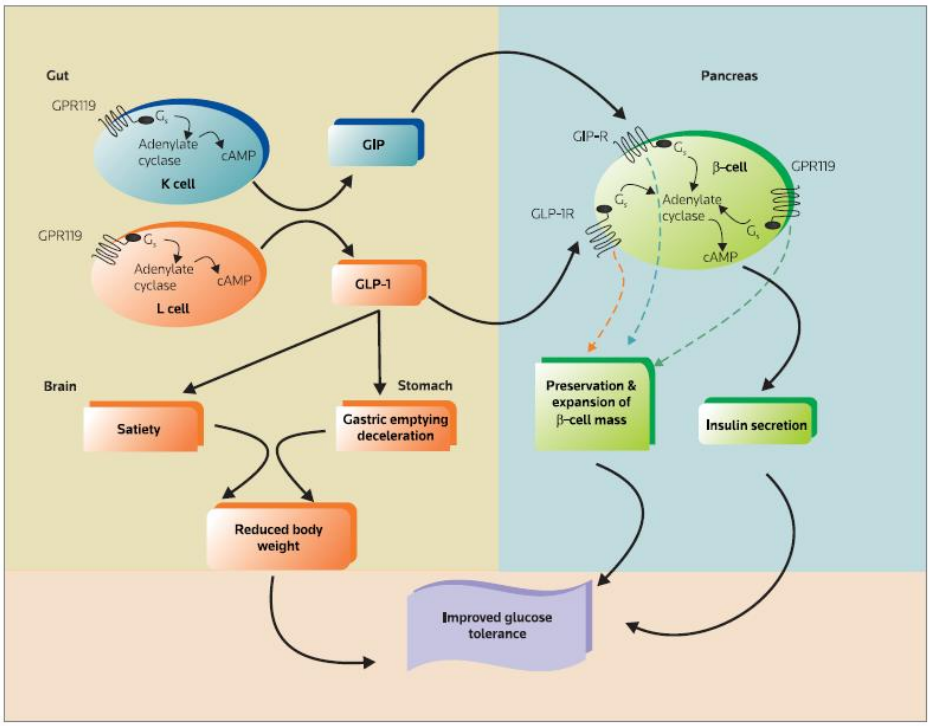
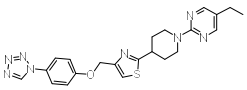
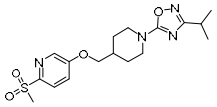
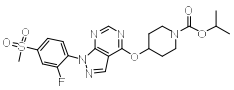
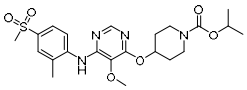
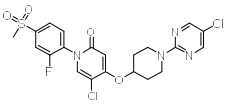


Figure 9. Physiological actions of GPR119. GPR119 is expressed on certain enteroendocrine cells (L and K cells) in the small intestine and by β-cells within the islets of Langerhans of the pancreas. In all three cell types, ligation of GPR119 by an agonist leads to the activation of adenylate cyclase and a rise in cAMP. This triggers the release of GLP-1, GIP or insulin from L, K and β cells, respectively. (Dhayal and Morgan 2010).

5. Synthetic small molecule GPR119 agonists

GPR119 agonists attracted the attention of the pharmaceutical industry due to the unique metabolic functions of GPR119. Great efforts have been carried out by several pharmaceutical companies to discover potent and selective GPR119 agonists. Following report of AR231453 (Chu et al. 2007), the first-generation GPR119 agonist developed by Arena Pharmaceuticals, many other pharmaceutical companies, including Astellas Pharma, GlaxoSmithKline, Merck, Metabolex, Pfizer, and Novartis have been pursuing GPR119 agonists as potential drug candidates for the treatment of T2DM. These small molecule GPR119 agonists have been observed to exert favorable effects on glucose homoeostasis, food intake and body weight, as well as dyslipidemia and protection of β-cells in animal models (Overton et al. 2008). Several synthetic small molecule GPR119 agonists have entered clinical trials based on their excellent preclinical performance. However, some clinical candidates failed to demonstrate effective glycemic control in humans due to weak efficacy or lack of durable blood glucose control after repeated dosing (Table 3) (Katz et al. 2012; Nunez et al. 2014; Ritter et al. 2106).

Table 3. Overview GPR119 agonists in clinical trials

Drugs	Company	Structure	Phase	Status
MBX-2982	CymaBay		Phase II	
PSN821	Astellas		Phase II	Discontinued
GSK1292263	GSK		Phase II	Discontinued
DS-8500	Daiichi Sankyo		Phase II	
LEZ763	Novartis		PhaseI/II	Discontinued
APD668	Arena		Phase I	Discontinued
APD597	Arena		Phase I	Discontinued
ZYG-19	Zydus Cadia		Phase I	
BMS903452	BMS		Phase I	

PURPOSE OF THIS STUDY

Among many pharmacological options for T2DM, GLP-1-based therapies including GLP-1 analogs and DPP-IV inhibitors are becoming increasingly popular because of their therapeutic benefits and safety (Drucker 2006). However, the efficacy of DPP-IV inhibitors is modest at best, as their actions are dependent on endogenous GLP-1. Although GLP-1 analogs are more efficacious than DPP-IV inhibitors, they require daily injection (Morales 2011). These limitations have prompted the search for a new class of oral agents capable of stimulating GLP-1 release.

GPR119 potentiates the release of GLP-1 in intestinal L cells while promoting GSIS in pancreatic β-cells. The combination of increasing endogenous GLP-1 release by GPR119 agonist and blocking its degradation by DPP-IV inhibitor could augment plasma active GLP-1 level. Thus, GPR119 agonists represent a potential new class of anti-diabetic agents which, in combination with DPP-IV inhibitors, may provide optimal oral GLP-1 based therapy. The present study identified a novel GPR119 agonist, YH18421 and validated its potential to treat T2DM alone or in combination with DPP-IV inhibitors.

MATERIALS AND METHODS

1. Chemicals

YH18421, clinical GPR119 agonists (MBX2982, JNJ38431055 and GSK1292263) and DPP-IV inhibitors (linagliptin and sitagliptin) were synthesized in-house at Yuhan R&D Institute (Korea). Compounds were dissolved in DMSO and diluted with cell culture media for *in vitro* cell based assays.

2. Cell culture

Human GPR119-CRE-*bla* CHO-K1 stable cell line (Invitrogen, USA) was cultured in DMEM (high glucose) media containing 10% fetal bovine serum, 0.1 mM NEAA (non-essential amino acids), 25 mM HEPES (pH 7.3), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 ug/ml zeocin and 600 ug/ml hygromycin (Invitrogen, USA). Hamster insulinoma cell line HIT-T15 (ATCC, USA) was cultured in Ham's F12K medium with 10% horse serum, 2.5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). Murine L cell line GLUTag (a gift from Dr. Hee Sook Jeon, Gacheon University, Korea) was cultured in DMEM (low glucose) with 10%

fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). Murine C2C12 skeletal muscle cells (ATCC, USA) cells were cultured in DMEM (high glucose) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). Rat H9c2 cardiac myoblast cells (ATCC, USA) were cultured in DMEM (high glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). Human hepG2 hepatocellular carcinoma cells (ATCC, USA) were cultured in EMEM (high glucose) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). All cell lines were cultured in 5 % CO_2 in a humidified atmosphere at $37^{O}C$.

3. Animals

All of the *in vivo* animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Yuhan Corporation. All animals were housed under a 12 h/12 h light/dark cycle in free feeding conditions, in temperature and humidity controlled rooms. Normal C57BL/6J male mice were obtained from Orient BIO Inc. (Korea).

To generate diet- induced obesity (DIO) mice, 5 week old-normal C57BL/6J mice were fed with high-fat chow (Kcal 60 % fat; D12492, Research Diet, USA) for 12 weeks. The mice that underwent a high-fat diet were checked for body weight, and mice which were over 40 g in body weight were considered as a DIO mouse model. Within studies in this article, animals were given a vehicle of 0.5 % methylcellulose (MC; Sigma-Aldrich, USA) in distilled water or test drugs suspension. For the study of body weight control, obese DIO mice are generated by long-term high-fat chow diet for 6 months and mice with the body weight of more than 50 g were considered as an obese DIO mouse model.

ob/ob mice were obtained from Central Lab. Animal Inc. (Korea). All animals were randomized into each group based on similar body weight and blood glucose.

4. cAMP accumulation assay

GPR119-CRE-*bla* CHO-K1 cells contain the human GPR 119 gene under the control of a doxycycline inducible system, stably integrated into the CRE-*bla* CHO-K1 cell line. CRE-*bla* CHO-K1 contains a beta-lactamase reporter gene under control of a CRE

response element stably integrated into CHO-K1 cells. Addition of doxycycline to these cells allows for GPR119 expression and subsequent assay for activity. GPR119-CRE-*bla* CHO-K1 cells were seeded onto 384-well plates (Thermo Scientific Nunc, USA) at a density of 10,000 cells/well and doxycycline (1 ug/ml) was added to induce GPR119 expression. After overnight incubation, the cells were exposed to YH18421 at various concentrations or vehicle (0.1 % DMSO). Following 5-hr incubation, cytoplasmic cAMP levels were measured using GeneBLAzer® assay technology following the manufacturer's instructions (Invitrogen, USA). The core of the GeneBLAzer® assay technology is a fluorescent detection of beta-lactamase reporter gene activity using a fluorescence resonance energy transfer (FRET)-enabled substrate that generates a ratiometric cAMP response. Three replicates for each concentration were used and the data are represented as % fold activation relative to the vehicle control.

5. Cell viability assay

GLUTag, HIT-T15, C2C12, H9c2 and hepG2 cells were seeded onto 96 well plates (Thermo Scientific Nunc, USA) at a density of 20,000, 30,000, 20,000, 10,000, and 20,000 cells/well, respectively and

incubated for 1 day. Cells were treated with YH18421 or vehicle (0.1 % DMSO) and further incubated for 2 days. Cell viability was determined via 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma, USA) colorimetric assay. In this assay metabolically viable active cells convert MTT to formazan via the cleavage of tetrazolium ring in active mitochondria (Mosmann, 1983). Thus reduced cell viability due to decreased mitochondrial activity is reflected by reduced absorbance at 570 nm. Following compound treatment period, the MTT dye was added directly to the treatment medium and incubated for an additional 4 hours. Media were then removed and DMSO was added to solubilize formazan. Absorbance was then read using the spectrophotometer (Molecular device, USA) at the wavelength of 570 nm.

6. GLP-1 secretion assay

GLUTag cells were seeded onto 48-well plates (Thermo Scientific Nunc, USA) at a density of 100,000 cells/well and then cultured for 2 days. On the day of the experiment, the cells were washed twice with serum-free DMEM media (low glucose) and were treated with the desired concentrations of YH18421 or vehicle (0.1 %

DMSO) in the same media at 37°C for 2 hr. GLP-1 secreted into the supernatant was then collected and clarified by centrifugation for 5 min. GLP-1 concentration in the supernatant was determined by an ELISA method using an GLP-1 ELISA kit (Shibayaki, Japan) following the manufacturer's instructions. Three replicates for each concentration of YH18421 were used.

7. Insulin secretion assay

HIT-T15 cells were seeded onto 48-well plates (Thermo Scientific Nunc, USA) at a density of 200,000 cells/well and cultured for 2 days. On the day of the experiment, the cells were washed twice with Krebs Ringer bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.16 mM MgCl2, 10 mM HEPES, 2.5 mM CaCl2, 25.5 mM NaHCO3, 0.2 % BSA, pH 7.4) and preincubated for 60 min at 37°C with the same buffer containing 5 mM glucose. After preincubation, cells were stimulated with the desired concentrations of YH18421 or vehicle (0.1 % DMSO) in Krebs Ringer bicarbonate buffer containing 15 mM glucose at 37°C for 60 min. Insulin secreted supernatant was then collected and clarified by centrifugation for 5 min. Insulin content in the supernatant was determined by an ELISA

method using an insulin ELISA kit (Mercodia, Sweden) following the manufacturer's instructions. Three replicates for each concentration of YH18421 were used.

8. C2C12 and H9c2 cell treatments

C2C12 and H9c2 cells were seeded onto extracellular matrix (Sigma−Aldrich, USA) coated 6-well tissue culture plates (Thermo Scientific Nunc, USA) at a density of 40,000 and 80,000 cells/well, respectively. At about 70% confluency, growth medium was replaced with DMEM supplemented with 2% horse serum, 1% penicillin streptomycin, and 0.5% amphotericin B to induce myoblast fusion, leading to the formation of differentiated myotubes. Cells were differentiated for 4 days with a change of medium every other day. Myotubes were pre-incubated with DMEM containing 0.1% bovine serum albumin for 2 hr and then exposed to YH18421 or vehicle (0.1 % DMSO) in DMEM containing 50 μM fatty acid free bovine serum albumin for 6 hr.

9. Quantitative real time PCR

Total RNA was extracted from myotubes using RNeasy Mini Kit

(Qiagen, Germany), following the manufacturer's instructions. Next, the RNA samples were converted to cDNA using the AffinityScript cDNA Synthesis Kit (Agilent Technologies, USA) with random primers. The sample was incubated at 25^{o}C for 5 min followed by 15 min at 42 ^{o}C. The reaction was terminated by heating the sample to 95 ^{o}C for 5 min.

The quantitative real time PCR was performed using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA) and the Stratagene Mx3005P QPCR System (Agilent Technologies, USA). 2uL of each cDNA sample was mixed with 1uL 200 pM forward primer, 1uL 200 pM reverse primer, and 12.5 ul SYBR Green PCR Master Mix. Quantitative real time PCR was initiated via heating at 95°C for 3 min before being ran for 40 or 50 cycles of 95°C for 15 s and 60 °C for 45 s for annealing and extension. Forward and reverse oligonucleotide primers for each gene are shown in Table 4. The data were analyzed using the MxPro QPCR Software. Relative changes in mRNA abundance of each gene product was normalized to GAPDH gene product.

Table 4. Primers for quantitative real-time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
AMPKα2	ACTCTGCTGATGCACATGCT	AGGGGTCTTCAGGAAAGAGG
PPARα	TGTCGAATATGTGGGGACAA	ACTTGTCGTACGCCAGCTTT
PGC-1α	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC
SOCS3	CTTCAGCTCCAAGAGCGAGT	AGCTGTCGCGGATAAGAAAG
GAPDH	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA

10. CYP3A4 induction assay

Plasmids. CYP3A4-PXRE-Luciferase reporter plasmid containing the proximal promoter (–362/+53) and distal promotor (–7836/–7208) inserted in pGL3 Basic vector (Promega, USA) was prepared as described (Tirona et al. 2003). The PXR expression plasmid was prepared by PCR from liver cDNA (Clontech, USA) using forward (5′-GAACATGGAGGTGAGACCCAAAGAAAGC-3′) and reverse (5′-TCAGCTACCTGTGATGCCGAACAACTC-3′) primers and subsequent cloning into pIRES expression vector (Invitrogen, USA). All expression plasmids were sequence verified.

Transient transfection assays. HepG2 cells were seeded into 96-well plates at a density of 20,000 cells/well. Following day, cells were transfected with 100 ng/well of luciferase reporter plasmid, 10 ng/well of pRL-TK (Promega, USA) vector (to normalize transfection yield) and 100 ng/well of PXR expression plasmid mixed with lipofectamine and Opti-MEM (Invitrogen, USA). After 3 hr, transfection mixture was removed and cells were incubated with rifampicine and YH18421 or vehicle (0.1 % DMSO). Luciferase reporter activities were measured with the dual luciferase reporter assay kit (Promega, USA) following the manufacturer's instructions.

11. Efficacy of single administration of YH18421 in normal mice

Acute efficacy of YH18421 was evaluated by oral glucose tolerance test (OGTT) with overnight fasted 8-week-old male C57BL/6J mice. After 30 min oral administration of 0.5% methyl-cellulose (vehicle) or YH18421, a glucose bolus was given orally at a dose of 2 g/kg/10 ml, and blood samples were collected from tail veins after 0, 5, 15, 30, 60, and 120 min. Plasma glucose was immediately determined using the Glucose sensor & strip system (GlucoDr^{+}, Allmedicus, Korea). The area under the curve (AUC) was calculated for statistical analysis in the OGTT experiment.

For plasma GLP-1, insulin and PYY pharmacodynamic studies, YH18421 was administered orally to fasted C57BL/6J mice. After 30 min, glucose bolus of 2 g/kg/10 ml was administered orally and blood was collected in heparinized blood collection tubes. Plasma samples were obtained by centrifugation and assayed for insulin using mouse insulin ELISA kit (Mercodia, Sweden) following the manufacturer's instructions. Plasma samples were assayed for total and active GLP-1 using mouse total GLP-1 ELISA kit (EMD Millipore, USA) and active GLP-1 ELISA kit (Shibayaki, Japan), respectively, following the manufacturer's instructions. Plasma samples were assayed for PYY

using mouse PYY ELISA kit (RayBiotech, USA) following the manufacturer's instructions.

12. Efficacy of repeated administrations of YH18421 in DIO mice

For the evaluation of chronic glycemic control, 17-week-old male DIO mice were orally administered 0.5% methyl-cellulose (vehicle) or YH18421 once daily for 4 weeks. On day1 and 28, efficacy of YH18421 was examined by oral glucose tolerance test (OGTT) with overnight fasted DIO mice. At 30 min after administration of YH18421, glucose bolus was given orally at a dose of 2 g/kg/10 ml, and blood glucose levels were monitored by tail snapping at -30, 0, 15, 30, 60, 90, 120, and 180 minutes after glucose load. Blood glucose levels were immediately determined using the Glucose sensor & strip system (GlucoDr^{+}, Allmedicus, South Korea). The area under the curve (AUC) was calculated for statistical analysis in the OGTT experiment.

For the study of body weight, long-term (6 months) high-fat chow diet fed DIO mice were orally given 0.5% methyl-cellulose (vehicle) or YH18421 once daily for 4 weeks. During the treatment period, the body weight of each animal was measured twice per week. Food intake of each group was measured weekly.

13. Efficacy of repeated administrations of YH18421 in *ob/ob* mice

For the evaluation of chronic glycemic control, 12-week-old male *ob/ob* mice were orally administered 0.5% methyl-cellulose (vehicle) or YH18421 once daily for 4 weeks. On day1 and 28, efficacy of YH18421 was examined by oral glucose tolerance test (OGTT) with overnight fasted *ob/ob* mice. At 30 min after administration of YH18421, glucose bolus was given orally at a dose of 2 g/kg/10 ml, and blood glucose levels were monitored by tail snapping at -30, 0, 15, 30, 60, 90, 120, and 180 minutes later glucose load. Blood glucose levels were immediately determined using the Glucose sensor & strip system (GlucoDr^{+}, Allmedicus, Korea). The area under the curve (AUC) was calculated for statistical analysis in the OGTT experiment.

14. Pharmacokinetics of YH18421 in normal mice

8-week-old male C57BL/6J mice were fasted overnight and orally administered YH18421 as suspension in 0.5% methyl cellulose at 1 mg/kg and 10 mg/kg. Blood samples were collected via inferior vena cava under anesthesia at 0.5, 1, 2, 4, 7, and 24 hr post-dose. Plasma samples were taken from the blood samples after centrifugation and proteins were precipitated to extract YH18421. The plasma

concentrations of YH18421 were determined by LC-MS/MS analysis. The pharmacokinetic analysis was calculated by a non-compartmental analysis using WinNonlin® version 6.3 (Pharsight Corporation, USA).

The pharmacokinetic parameters of YH18421 are as follows; maximum observed plasma concentration (C_{max}), time to reach maximum observed plasma concentration (t_{max}), area under the plasma concentration-time curve from time zero to the time of the last measurable concentration (AUC_{0-24hr}), and area under the plasma concentration-time curve extrapolated to infinity (AUC_{inf}), apparent terminal half-life (*t*1/2).

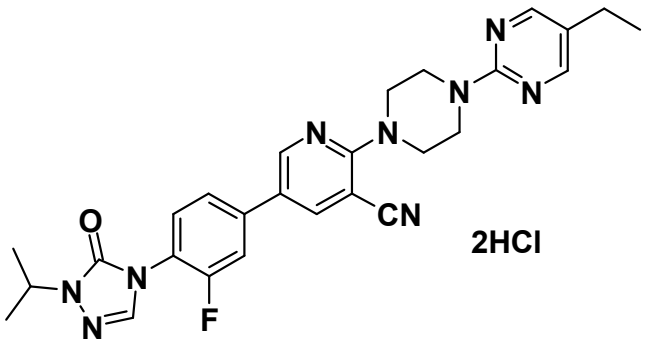
15. Statistical analysis

Values were expressed as the mean ± SEM. Student's t-test was used to compare two groups, and multiple group comparisons were performed using one-way ANOVA with a post hoc analysis (Tukey or Dunnett's multiple comparison test). Statistical analyses were done using Prism software (Graphpad, USA), and a p value of < 0.05 was considered to be statistically significant.

RESULTS

1. Identification of YH18421

In order to identify novel GPR119 agonists, about 8,000 compounds were selected from commercial libraries and Yuhan in-house library through a ligand-based virtual screening approaches. A cell based high-throughput screening (HTS) assay was established based on the signal transduction pathway of GPR119. Selected 8,000 compounds were screened, and several hits with novel chemical scaffolds were identified. Based on these hit scaffolds, approximately 3,000 derivatives were rationally designed, synthesized, and screened for their ability to specifically activate human GPR119 in a cell based cAMP assay. Further chemical lead optimization study with *in vitro* cell based functional assays and *in vivo* acute mouse OGTT evaluations led to identification of a novel, potent, and specific human GPR119 agonist, YH18421. YH18421 is composed of 4-[4-(6-Piperazin-1-yl-pyridin-3-yl)-phenyl]-2,4-dihydro-[1,2,4]triazol-3-one derivative (Figure 10) and patented (Patent No. WO2014-175621).



YH18421

Figure 10. Chemical structure of YH18421.

2. YH18421 increases GPR119 induced intracellular cAMP accumulation

The specific agonistic activity of human GPR119 by YH18421 was determined by assessing intracellular cAMP levels in CHO-K1 cells stably expressing the human GPR119 receptor under the control of a doxycycline inducible system. YH18421 potently induced intracellular cAMP accumulation in a concentration-dependent manner. YH18421 had no effect on cAMP accumulation in CHO-K1 cells cultured without a doxycycline thus not expressing the human GPR119 receptor (Figure 11A). Next, the potency of YH18421 was compared with that of clinical GPR119 agonists (MBX2982, JNJ38431055, and GSK1292263). The EC_{50} values of each compound were determined to be 2.2 nM for YH18421, 43.5 nM for MBX2982, 43.9 nM for GSK1292263, and 40.2 nM for JNJ38431055, respetively. YH18421 activated human GPR119 approximately 20 fold more potently than clinical GPR119 agonists (Figure 11B).

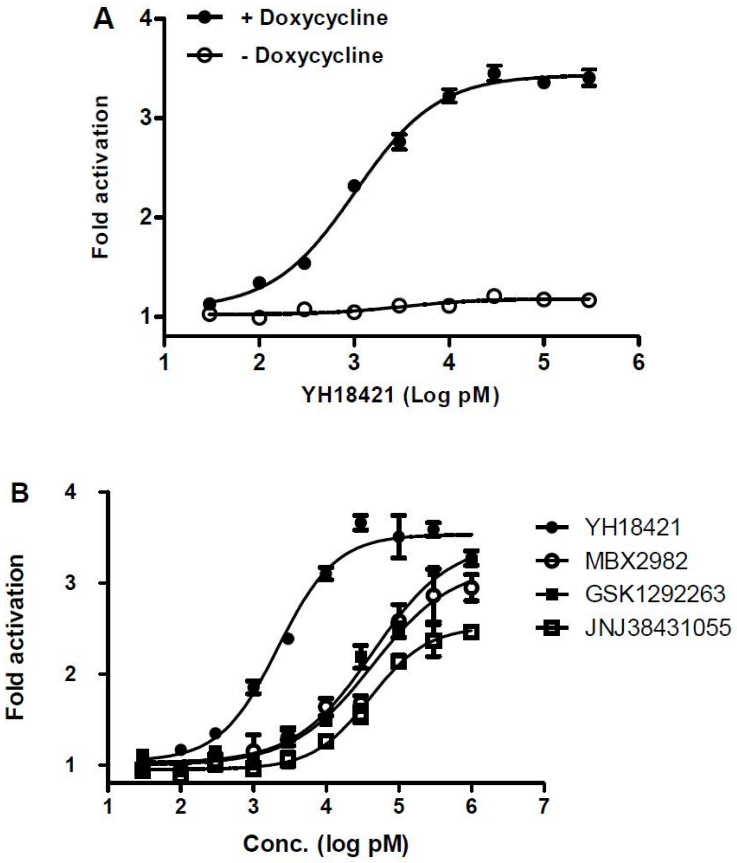


Figure 11. GPR119 activation by YH18421. CHO-K1 cells stably expressing human GPR119 were incubated with YH18421 and clinical GPR119 agonists for 5 hr. Intracellular cAMP levels were measured with CRE-beta-lactamase reporter gene. A, Activation of GPR119 by YH18421. B, Comparison of YH18421 with clinical GPR119 agonists. Triplicates for each concentration were used and the data are represented as fold activation relative to control.

3. YH18421 is a orthosteric GPR119 agonist for endogenous ligand

To determine whether YH18421 could orthosterically or allosterically modulate the activity of OEA, a endogenous ligand of GPR119, 3, 10, and 30 μM OEA were added to various concentrations of YH18421. Orthosteric agonist binds in the same site on the receptor as the endogenous ligand (the orthosteric site), whereas allosteric agonist binds to a site that are distinct from the orthosteric site. It was found that OEA was able to increase basal responses elicited by YH18421 without affecting its potency (Figures 12). Such an increase of basal signal is due to the activity of OEA on GPR119 alone. EC_{50} values of YH18421 with each concentrations OEA were 2.9 nM for 30 μM OEA, 2.7 nM for 10 μM OEA, 3.0 nM for 3 μM OEA, and 2.9 nM for YH18421 alone, respectively. The EC_{50} of YH18421 did not change significantly with the addition of varying concentrations of OEA. The lack of a shift in potency (no significant change of EC_{50}) of YH18421 with the addition of OEA suggests that YH18421 and OEA either bind to the same site of GPR119 receptor or their binding areas are somehow overlaps.

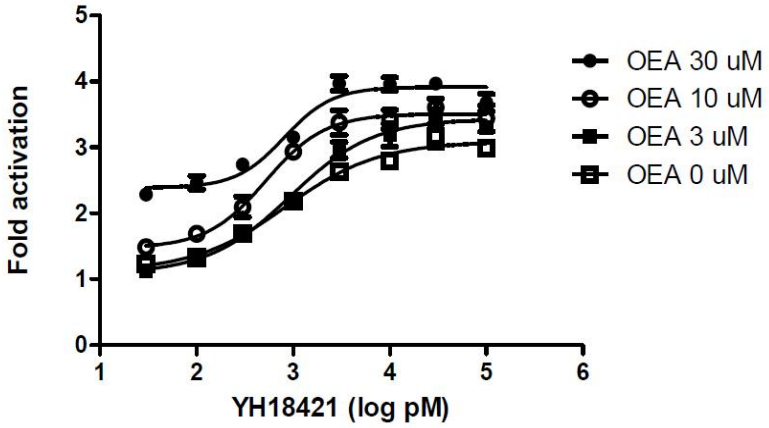
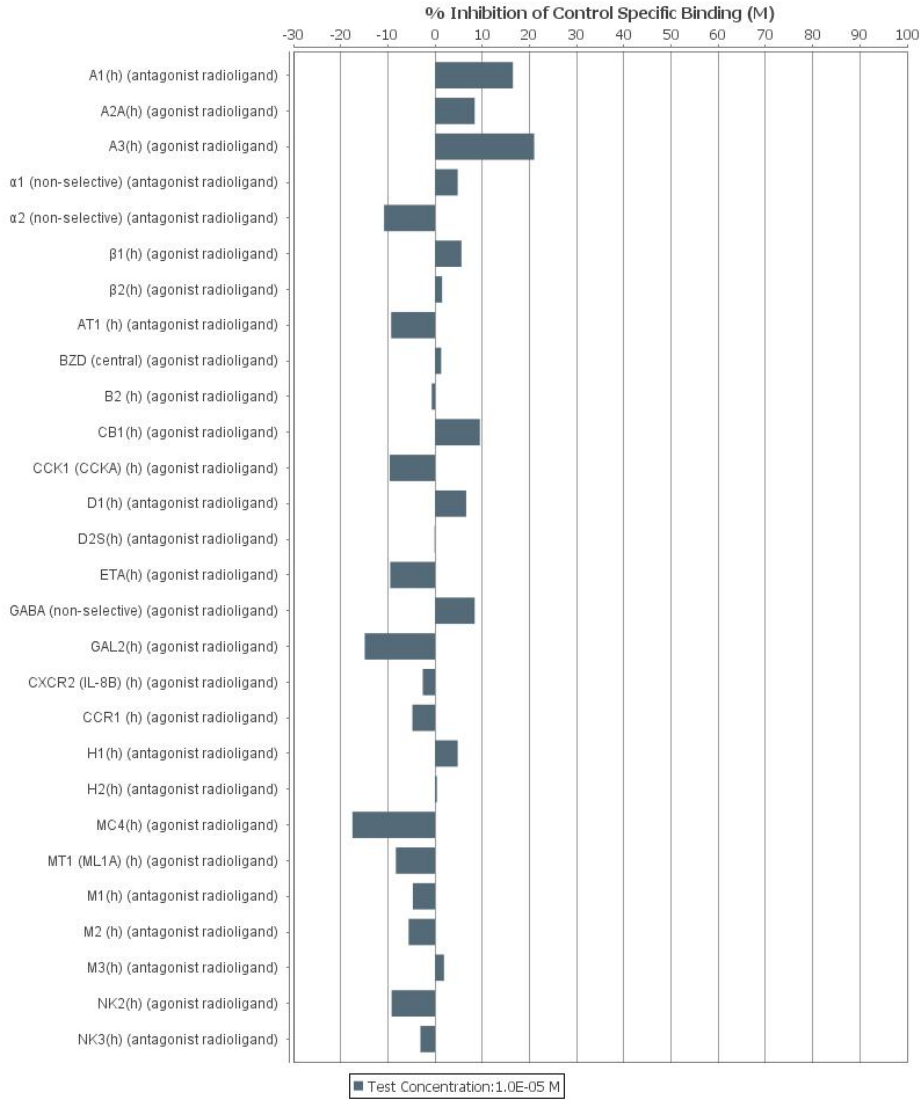


Figure 12. GPR119 activation by combination of YH18421 and OEA. CHO-K1 cells stably expressing human GPR119 were incubated with various concentrations of YH18421 and OEA for 5 hr. Intracellular cAMP levels were measured with CRE-beta-lactamase reporter gene. Triplicates for each concentration were used and the data are represented as fold activation relative to control.

4. YH18421 is a selective agonist of GPR119

To identify possible off target GPCR activities of YH18421, radioligand binding assays against 55 selected off targets were performed by Cerep company (France). These 55 off target panel was composed of 47 GPCRs, 5 ion channels and 3 transporters and recommended by Cerep company as a minimal panel that should provide a broad early assessment of the potential off target effects of a compound. In each binding assay, the respective reference compound for each GPCRs was tested concurrently with 10 μM YH18421. Results showing an inhibition or stimulation for assays run in basal conditions higher than 50% are considered to represent significant binding interaction of the test compound with each target. YH18421 showed no significant activity against a panel of 45 GPCRs and 10 non-GPCR off targets (Figure 13). Together with cAMP accumulation data in figure 11 and 12, these results demonstrate that YH18421 is a highly potent and selective agonist of human GPR119.


% Inhibition of Control Specific Binding (M)
-30
-20
-10
0
10
20
30
40
50
60
70
80
90
100
A1(h) (antagonist radioligand)
A2A(h) (agonist radioligand)
A3(h) (agonist radioligand)
α1 (non-selective) (antagonist radioligand)
α2 (non-selective) (antagonist radioligand)
β1(h) (agonist radioligand)
β2(h) (agonist radioligand)
AT1 (h) (antagonist radioligand)
BZD (central) (agonist radioligand)
B2 (h) (agonist radioligand)
CB1(h) (agonist radioligand)
CCK1 (CCKA) (h) (agonist radioligand)
D1(h) (antagonist radioligand)
D2S(h) (antagonist radioligand)
ETA(h) (agonist radioligand)
GABA (non-selective) (agonist radioligand)
GAL2(h) (agonist radioligand)
CXCR2 (IL-8B) (h) (agonist radioligand)
CCR1 (h) (agonist radioligand)
H1(h) (antagonist radioligand)
H2(h) (antagonist radioligand)
MC4(h) (agonist radioligand)
MT1 (ML1A) (h) (agonist radioligand)
M1(h) (antagonist radioligand)
M2 (h) (antagonist radioligand)
M3(h) (antagonist radioligand)
NK2(h) (agonist radioligand)
NK3(h) (antagonist radioligand)
Test Concentration:1.0E-05 M

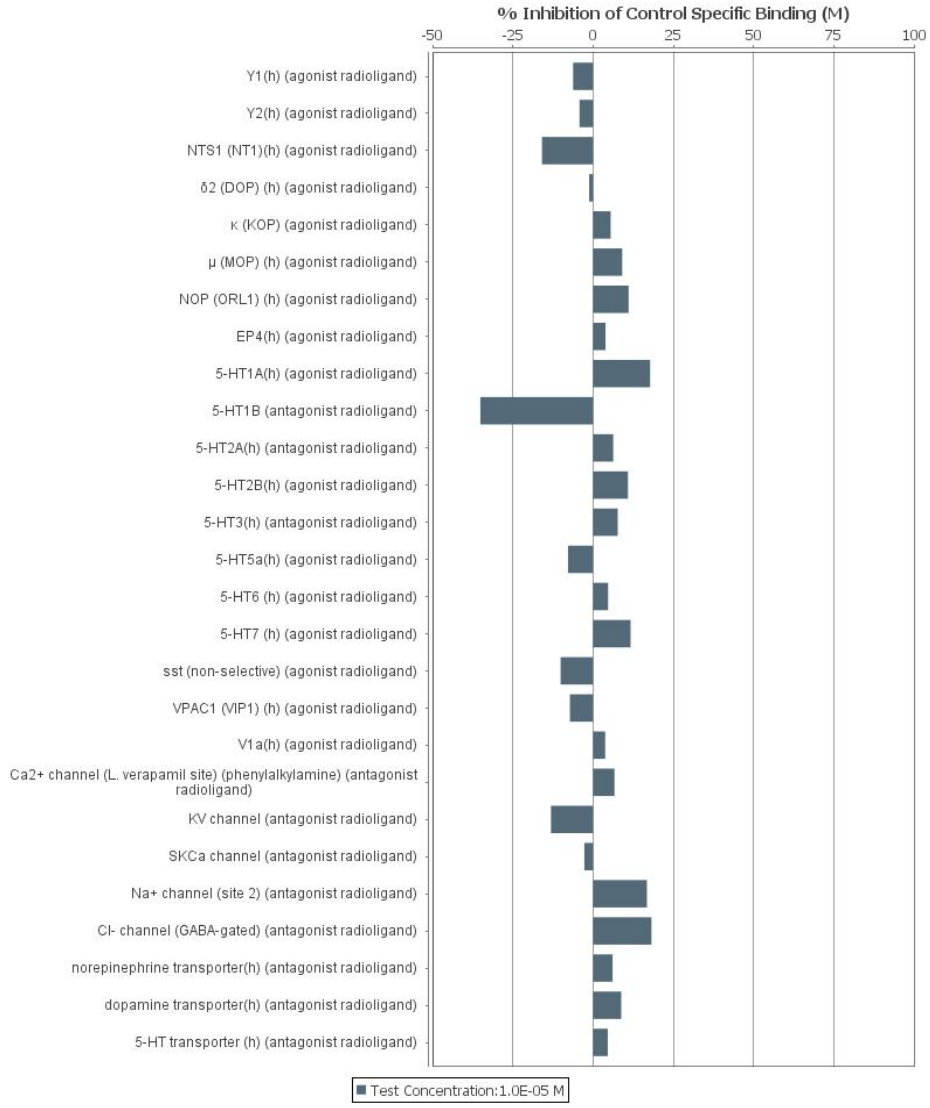


Figure 13. Inhibition of specific binding of reference control against off target panel by YH18421.

5. YH18421 stimulates L cell GLP-1 and β cell insulin secretion

To confirm that YH18421 has dual functional effects on intestinal L cells and pancreatic β cells, GLP-1 and insulin secretion were determined from the GLUTag mouse L cell line and HIT-T15 hamster β cell line. These two cell lines are known to express GPR119 endogenously (Overton et al. 2008; Lauffer et al. 2009). First, cytotoxic effects of YH18421 on GLUTag and HIT-T15 cells were examined. Upto 30 μM (maximum soluble concentration in cell culture media), YH18421 didn't affect the viability of GLUTag and HIT-T15 cells (Figure 14). Then, stimulatory effect of YH18421 on the secretion of GLP-1 and insulin from GLUTag and HIT-T15 cells was determined. YH18421 elicited dose-dependent increases GLP-1 and insulin release from the GLUTag and HIT-T15 cells, respectively (Figure 15). These results confirm that YH18421 mediates dual functional activity of both GLP-1 and insulin secretion at the cellular level.

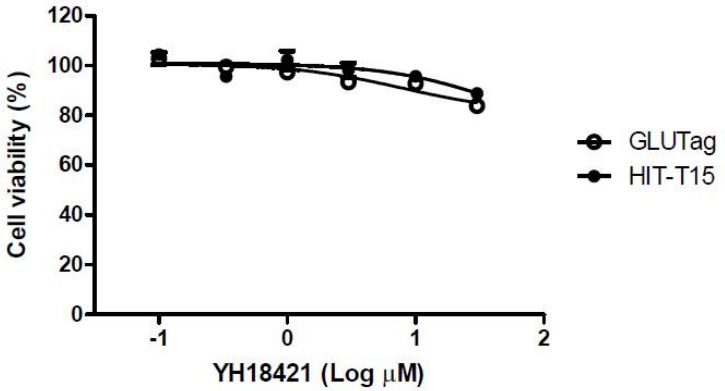


Figure 14. Effect of YH18421 on the viability of GLUTag and HIT-T15 cells. Cells were incubated with YH18421 for 2 days. Cell viability was measured with MTT colorimetric method. Triplicates for each concentration were used and the results are presented as the mean ± SEM.

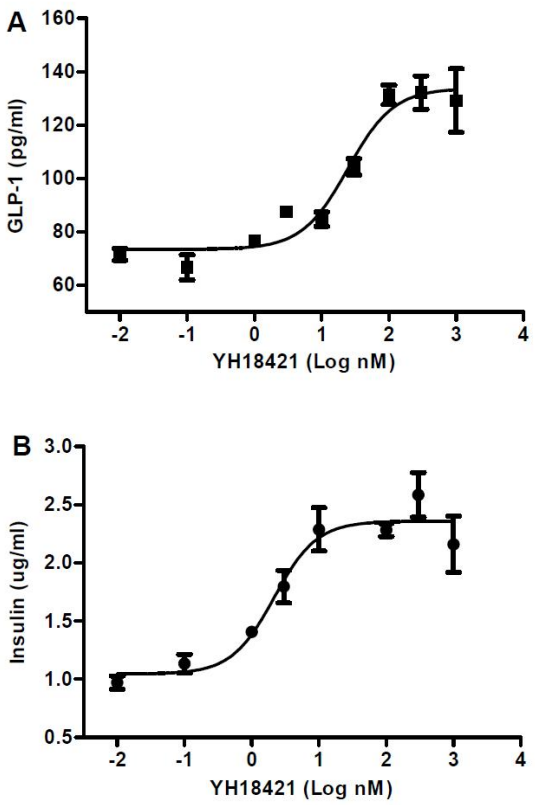


Figure 15. Stimulation of GLP-1 and insulin secretion by YH18421. A, Measurement of GLP-1 secretion from GLUTag cells. Cells were incubated with YH18421 for 2 hr. GLP-1 in the supernatant was measured with ELISA method. B, Measurement of insulin secretion from HIT-T15 cells. Cells were incubated with YH18421 for 1 hr. Insulin in the supernatant was measured with ELISA method. Triplicates for each concentration were used and the results are presented as the mean ± SEM.

Some insulin secretagogue anti-diabetic drugs such as sulfonylureas can cause hypoglycemia side effect because their insulinotrophic actions are not glucose concentration dependent (Sola et al. 2015). Thus it is important for insulinotrophic agents to stimulate insulin secretion in a glucose dependent manner. The effect of YH18421 on GSIS (glucose-stimulated insulin secretion) was examined at various glucose concentrations. Upon treatment of 100 nM YH18421 (effective concentration for maximum insulin secretion) in the HIT-T15 cells, insulin release was enhanced in glucose dependent manner, while it had no effect on cells incubated without glucose (Figure 16). These results suggest that YH18421 mediates glucose dependent insulin secretion and the risk of hypoglycemia can be minimized.

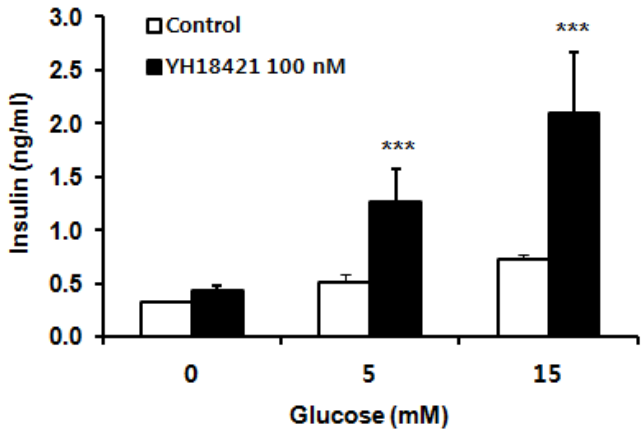
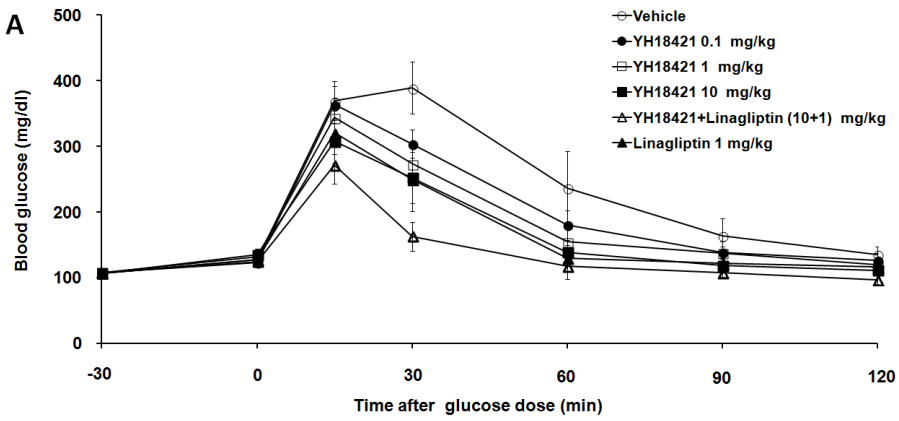


Figure 16. Glucose stimulated insulin secretion by YH18421. HIT-T15 cells were incubated with 100 nM YH18421 with 0, 5 and 15 mM glucose. After 1-hr incubation, insulin in the supernatant was measured with ELISA method. Triplicates for each concentration were used and the results are presented as the mean ± SEM. ***$P < 0.001$ versus control.

6. Acute YH18421 administration improves glucose tolerance in normal mice

After demonstrating that YH18421 specifically activated GPR119 and stimulated GLP-1 and insulin secretion *in vitro*, OGTT (oral glucose tolerance test) using normal mice was performed to evaluate the effects of YH18421 on acute glucose homeostasis *in vivo*. After 30 min oral administration of 0.1, 1 and 10 mg/kg YH18421, a glucose bolus was given orally at a dose of 2 g/kg/10 ml and blood samples were collected to analyze blood glucose concentration. Single oral administration of YH18421 dose-dependently lowered blood glucose levels during 2 hr following oral glucose dosing compared to the vehicle group as determined by the AUC (area under the curve) values. Combination of YH18421 and DPP-IV inhibitor was also administered to examine whether this regimen might result in additional blood glucose lowering efficacy. When 10 mg/kg YH18421 was dosed in combination with 1 mg/kg linagliptin (DPP-IV inhibitor), a significant and additive glucose lowering effect was observed compared to YH18421 or linagliptin alone (Figure 17).


A
Blood glucose (mg/dl)
500
400
300
200
100
0
-30
0
30
60
90
120
Time after glucose dose (min)
Vehicle
YH18421 0.1 mg/kg
YH18421 1 mg/kg
YH18421 10 mg/kg
YH18421+Linagliptin (10+1) mg/kg
Linagliptin 1 mg/kg

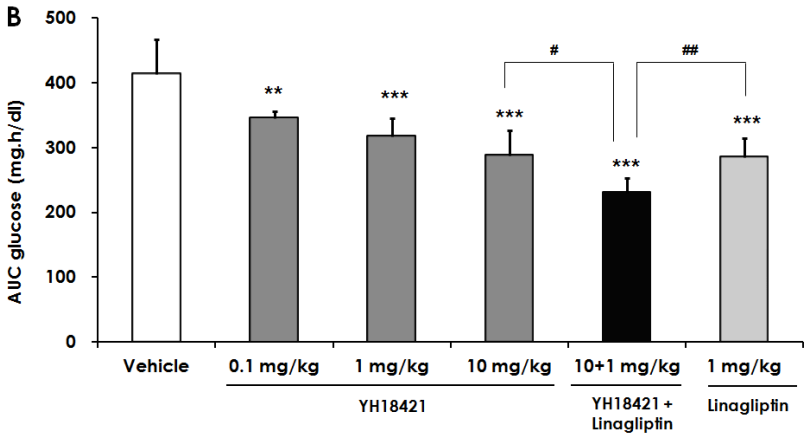
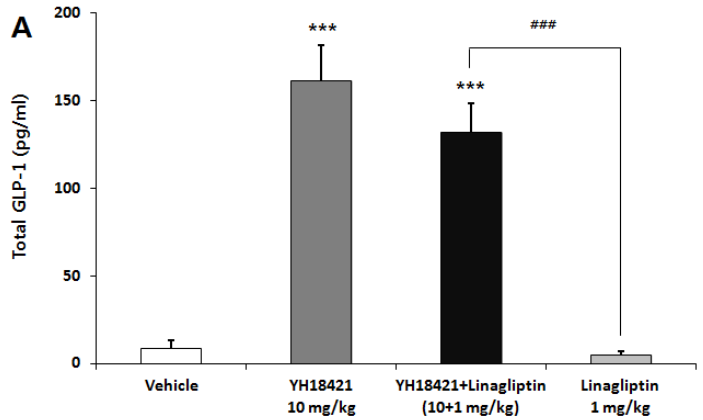

B
AUC glucose (mg.h/dl)
500
400
300
200
100
0
**
***
***
***
***
#
##
Vehicle
0.1 mg/kg
1 mg/kg
10 mg/kg
10+1 mg/kg
1 mg/kg
YH18421
YH18421 + Linagliptin
Linagliptin

Figure 17. Single YH18421 treatment improved acute oral glucose tolerance in normal mice (n=8). C57BL/6J mice were treated with vehicle or compounds 30 min before glucose bolus (2g/kg) and blood samples were collected over 2 hr. A**,** OGTT in mice treated with vehicle (0.5% MC, open circle), YH18421 (0.1 mg/kg, closed circle; 1 mg/kg, open square; 10 mg/kg, closed square), YH18421+linagliptin (10+1 mg/kg, open triangle) or linagliptin (1 mg/kg, closed triangle). B**,** The area under the plasma glucose concentration–time curve for 2 h ($AUC_{0-2\ h}$) in an OGTT. Results are presented as the mean ± SEM. $^{**}P < 0.01$,$^{***}P < 0.001$ versus vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between treatment groups being compared.

7. Acute YH18421 administration enhanced blood GLP-1 secretion in normal mice

After observation that YH18421 effectively lowered blood glucose level in normal mouse during OGTT, the pharmacodynamic effects of YH18421 on blood GLP-1 levels were examined with overnight-fasted mice following oral administration. Administration of 10 mg/kg YH18421 increased blood total GLP-1 and active GLP-1 levels by 17.3 and 3.0 fold compared to the vehicle control at 30 minutes after drug administration, respectively. Because GLP-1 has a very short half-life (~ 1 minute) *in vivo*, increase in active GLP-1 was thought to be much smaller than increase in total GLP-1. However, as seen in additive glycemic control during normal mouse OGTT, the combination of YH18421 with linagliptin (DPP-IV inhibitor) significantly elicited synergistic increases (23 fold *vs*. vehicle) in active GLP-1 level compared to YH18421 or linagliptin alone (Figure 18). These results demonstrate that combination of YH18421 with DPP-IV inhibitor is effective for maximizing blood active GLP-1 levels.


A
Total GLP-1 (pg/ml)
200
150
100
50
0
***
***
###
Vehicle
YH18421
10 mg/kg
YH18421+Linagliptin
(10+1 mg/kg)
Linagliptin
1 mg/kg

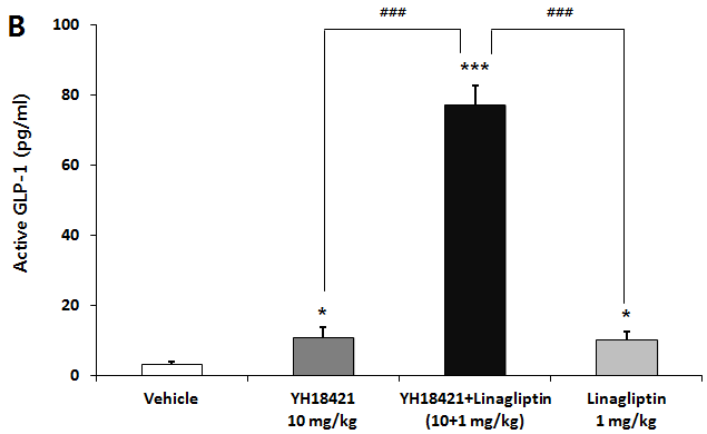
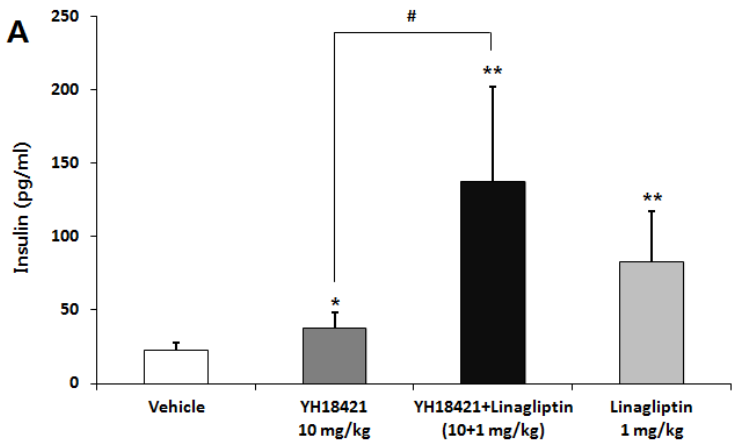

B
Active GLP-1 (pg/ml)
100
80
60
40
20
0
###
###
***
*
*
Vehicle
YH18421
10 mg/kg
YH18421+Linagliptin
(10+1 mg/kg)
Linagliptin
1 mg/kg

Figure 18. YH18421 enhanced blood GLP-1 release in normal mice (n=6). A, Blood total GLP-1 levels. C57BL/6J mice treated with vehicle (0.5% MC), YH18421 (10 mg/kg), linagliptin (1 mg/kg) or YH18421+linagliptin (10+1 mg/kg). Blood samples were collected at 30 min and plasma total GLP-1 levels were measured. B, Blood active GLP-1 levels. C57BL/6J mice treated with vehicle (0.5% MC), YH18421 (10 mg/kg), linagliptin (1 mg/kg) or YH18421+linagliptin (10+1 mg/kg). Blood samples were collected at 30 min and plasma active GLP-1 levels were measured. Results are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle, $^{\#\#\#}P < 0.001$ between treatment groups being compared.

8. Acute YH18421 administration enhances blood insulin and PYY secretion in normal mice

After observation that YH18421 effectively lowered blood glucose level in normal mouse during OGTT, the pharmacodynamic effects of YH18421 on blood insulin levels were examined with overnight-fasted mice following glucose administration. Administration of 10 mg/kg YH18421 increased blood insulin levels by 1.7 fold compared to the vehicle control. The combination of YH18421 with linagliptin significantly increased blood insulin levels in an additive manner compared to YH18421 alone (Figure 19A).

To investigate whether YH18421 also increases circulating PYY, a hypophagic gastrointestinal hormone, overnight-fasted mice were treated with YH18421 following glucose administration. Administration of 10 mg/kg YH18421 significantly increased plasma PYY levels by 3.6 fold compared to the vehicle group (Figure 19B). Since DPP-IV inhibitor does not affect endogenous PYY release, the effect of combined delivery of YH18421 with DPP-IV inhibitor on plasma PYY secretion was not investigated.


A
Insulin (pg/ml)
250
200
150
100
50
0
#
**
*
**
Vehicle
YH18421
10 mg/kg
YH18421+Linagliptin
(10+1 mg/kg)
Linagliptin
1 mg/kg

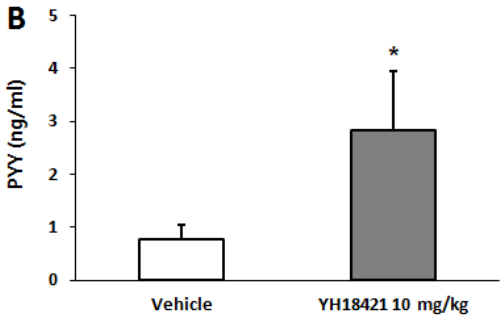
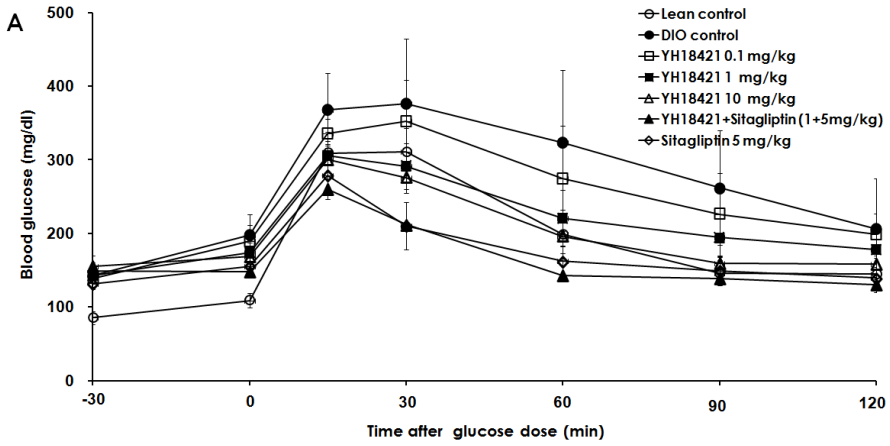

B
PYY (ng/ml)
5
4
3
2
1
0
*
Vehicle
YH18421 10 mg/kg

Figure 19. YH18421 enhanced blood insulin and PYY release in normal mice (n=6). A, Blood insulin levels. C57BL/6J mice treated with vehicle (0.5% MC), YH18421 (10 mg/kg), linagliptin (1 mg/kg) or YH18421+linagliptin (10+1 mg/kg). After 30 min, glucose bolus was given and blood samples were collected at 30 min. Insulin levels in plasma were measured. B, Blood PYY levels. C57BL/6J mice treated with vehicle (0.5% MC) or YH18421 (10 mg/kg). After 30 min, glucose bolus was given and blood samples were collected at 30 min and plasma PYY levels were measured. Results are presented as the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus vehicle, $^{\#}P < 0.05$ between treatment groups being compared.

9. Repeated administration of YH18421 improves chronic glucose tolerance in DIO mice

To evaluate the sustained efficacy of repeated administrations of YH18421, diabetic DIO (diet-induced obese) mouse model was generated. DIO mice were orally administered once daily with 0.1, 1 or 10 mg/kg YH18421 or vehicle for 4 weeks. An OGTT was performed on the day 1 and day 28 following YH18421 administration. On the day 1 OGTT, YH18421 dose-dependently lowered blood glucose levels compared to vehicle group (Figure 20). On the day 28 following YH18421 treatment, an OGTT was performed again to examine whether the efficacy of YH18421 on the day 1 could be maintained. YH18421 effectively improved glucose tolerance on day 28 with maximum efficacy observed at 1 mg/kg (Figure 21). The combination of 1 mg/kg YH18421 and 5 mg/kg sitagliptin (DPP-IV inhibitor) resulted in significant additive glycemic control compared to YH18421 alone. When compared to sitagliptin alone, the combination of YH18421 and sitagliptin tended to elicit greater reduction in blood glucose, though it was not statistically significant. These findings suggest that efficacy of YH1841 can be sustained in diabetic mouse.


A
Lean control
DIO control
YH18421 0.1 mg/kg
YH18421 1 mg/kg
YH18421 10 mg/kg
YH18421+Sitagliptin (1+5mg/kg)
Sitagliptin 5 mg/kg
Blood glucose (mg/dl)
500
400
300
200
100
0
-30
0
30
60
90
120
Time after glucose dose (min)

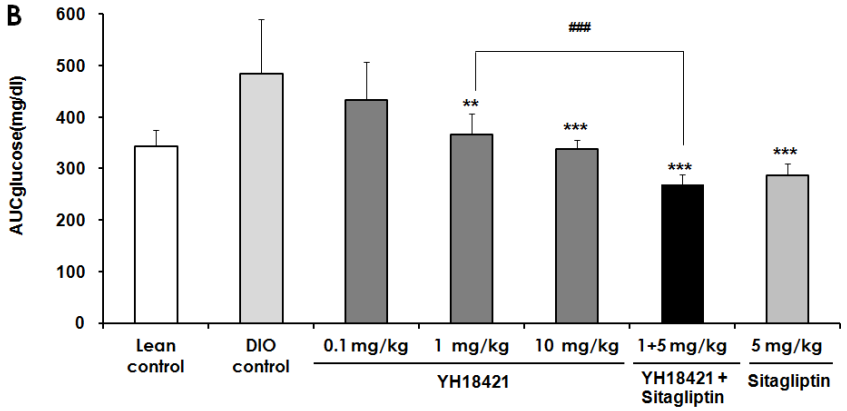
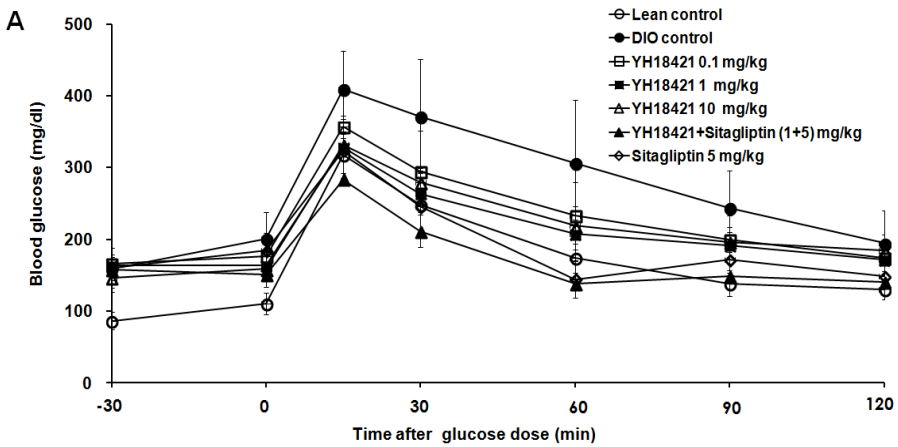

B
AUCglucose(mg/dl)
600
500
400
300
200
100
0
###
**
***
***
***
Lean control
DIO control
0.1 mg/kg
1 mg/kg
10 mg/kg
YH18421
1+5 mg/kg
YH18421 + Sitagliptin
5 mg/kg
Sitagliptin

Figure 20. YH18421 treatment improved acute glucose tolerance in DIO mice (n=8). DIO mice were treated with vehicle or compounds 30 min before glucose bolus (2 g/kg) and blood samples were collected over 2 hr. A, OGTT in DIO mice treated with vehicle (0.5% MC, closed circle), YH18421 (0.1 mg/kg, open square; 1 mg/kg, closed square; 10 mg/kg, open triangle), YH18421+sitagliptin (10+5 mg/kg, closed triangle) or sitagliptin (5 mg/kg, open diamond). B, The area under the plasma glucose concentration–time curve for 2 h ($AUC_{0-2\ h}$) in an OGTT. Results are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle, $^{\#\#\#}P < 0.001$ between treatment groups being compared.


A
Blood glucose (mg/dl)
500
400
300
200
100
0
-30
0
30
60
90
120
Time after glucose dose (min)
Lean control
DIO control
YH18421 0.1 mg/kg
YH18421 1 mg/kg
YH18421 10 mg/kg
YH18421+Sitagliptin (1+5) mg/kg
Sitagliptin 5 mg/kg

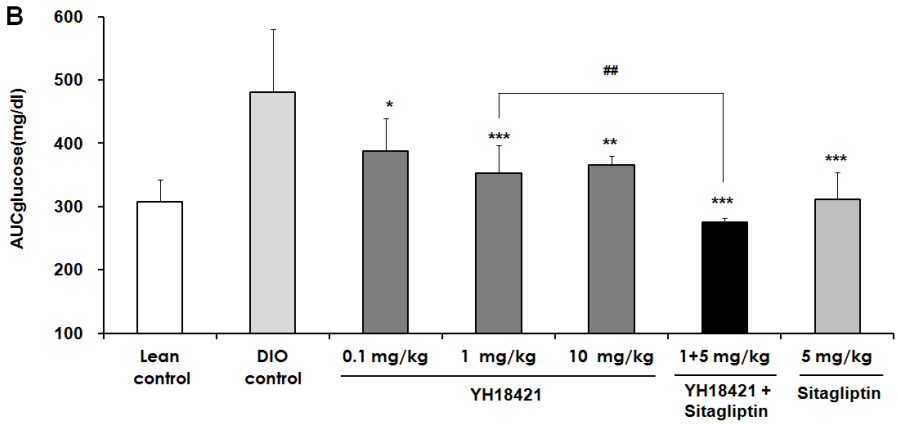
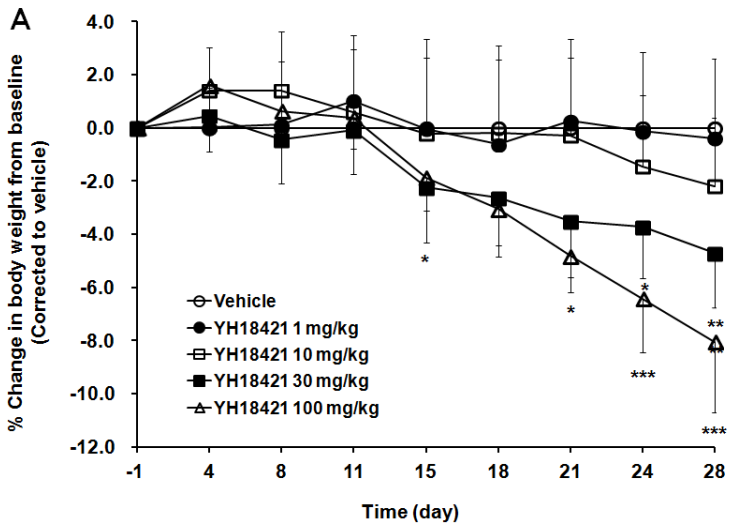

B
AUCglucose(mg/dl)
600
500
400
300
200
100
*
***
**
##
***
***
Lean control
DIO control
0.1 mg/kg
1 mg/kg
10 mg/kg
YH18421
1+5 mg/kg
YH18421 + Sitagliptin
5 mg/kg
Sitagliptin

Figure 21. Repeated YH18421 treatment improved chronic glucose tolerance in DIO mice (n=8). On the day 28 following daily dosing of YH18421**,** DIO mice were treated with vehicle or compounds 30 min before glucose bolus (2g/kg) and blood samples were collected over 2 hr. A, OGTT in DIO mice treated with vehicle (0.5% MC, closed circle), YH18421 (0.1 mg/kg, open square; 1 mg/kg, closed square; 10 mg/kg, open triangle), YH18421+sitagliptin (10+5 mg/kg, closed triangle) or sitagliptin (5 mg/kg, open diamond). B, The area under the plasma glucose concentration–time curve for 2 h ($AUC_{0-2\ h}$) in an OGTT. Results are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle, $^{\#\#}P < 0.01$ between treatment groups being compared.

10. Chronic administration of YH18421 inhibits weight gain in DIO mice

In a separate study using obesity model of the DIO mouse, the effect of YH18421 on body weight was investigated. Obesity model of DIO mouse was generated by feeding long-term high-fat chow diet for 6 months and the body weight of these animals reached more than 50 g. Obese DIO mice were orally administered once daily with 1, 10, 30 or 100 mg/kg YH18421 or vehicle for 4 weeks. YH18421 dose-dependently inhibited weight gain during the treatment period. Relative to changes in the vehicle group, administration of YH18421 at the higher dose of 100 mg/kg caused 8.1% weight loss on the day 28 of treatment (Figure 22A). To find out reason behind body weight loss by YH18421, changes in food intake in animals treated with YH18421 were examined. Cumulative food intake over the 28 days of administration was reduced with YH18421 treatment at 30 and 100 mg/kg relative to the vehicle control (Figure 22B). Statistical analysis for food intake was not done because average food intake was measured per cage, not individual animals.


A
% Change in body weight from baseline
(Corrected to vehicle)
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-1
4
8
11
15
18
21
24
28
Time (day)
Vehicle
YH18421 1 mg/kg
YH18421 10 mg/kg
YH18421 30 mg/kg
YH18421 100 mg/kg
*
*
*
**
***
***

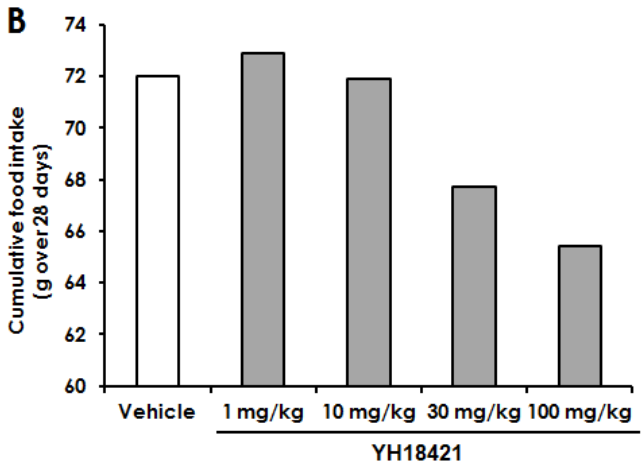

B
Cumulative food intake
(g over 28 days)
74
72
70
68
66
64
62
60
Vehicle
1 mg/kg
10 mg/kg
30 mg/kg
100 mg/kg
YH18421

Figure 22. Repeated YH18421 treatment inhibited body weight gain and food intake in obese DIO mice (n=8). A, Body weight change in DIO mice treated once daily with vehicle (0.5% MC, open circle) or YH18421 (1 mg/kg, closed circle; 10 mg/kg, open square; 30 mg/kg, closed square; 100 mg/kg, open triangle). Body weight expressed as a percentage change from baseline body weight and adjusted to vehicle controls. B, Cumulative food intake over the 28 days of treatment. Results are presented as the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus vehicle.

11. Combination YH18421 with DPP-IV inhibitor improves chronic glucose tolerance in *ob/ob* mice

To determine the sustained efficacy of YH18421 in another diabetic model, *ob/ob* mouse was treated with YH18421 for 4 weeks. The *ob/ob* mouse is a monogenic mutant mouse that eats excessively due to mutations in the gene responsible for the production of leptin and becomes profoundly obese (Lindström P 2007). The *ob/ob* mice, which achieve obesity earlier in life, generally accompany more severe form of hyperglycemia and diabetes than DIO mice (Rausch ME et al. 2008).

ob/ob mice were orally administered once daily with 1 mg/kg YH18421, YH18421+linagliptin (1+0.5 mg/kg), 0.5 mg/kg linagliptin or vehicle for 4 weeks. An OGTT was performed on the day 1 and 28 following compound administration. On the day 1 and 28 OGTT, either YH18421 or linagliptin alone failed to lower blood glucose levels compared to vehicle group. However, combined treatment of 1 mg/kg YH18421 and 0.5 mg/kg linagliptin effectively improved glucose tolerance (Figure 23, 24). These findings suggest that combination of YH18421 with DPP-IV inhibitor can be effective even in severe chronic diabetic disease model like *ob/ob* mouse.

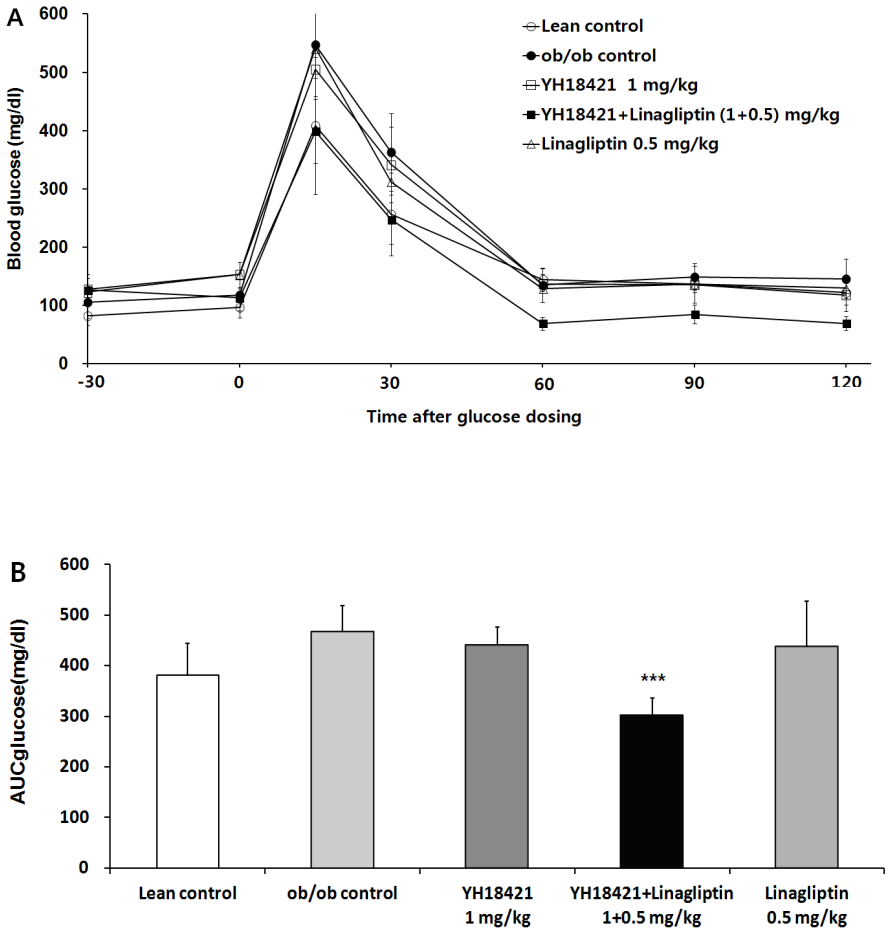
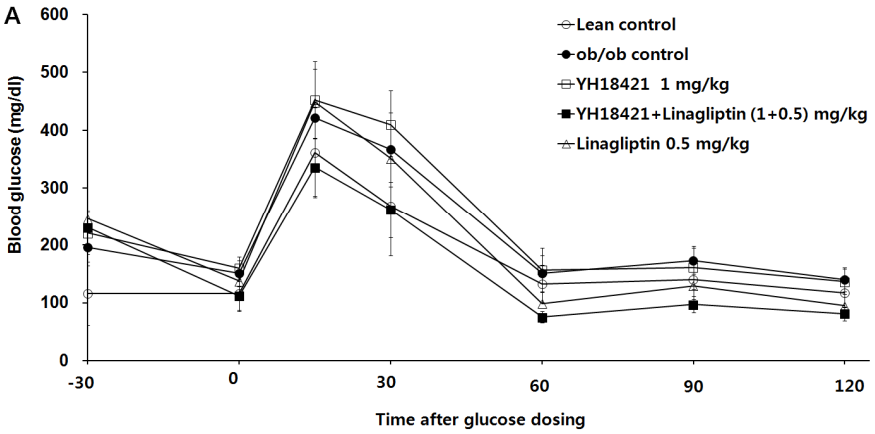

A
Blood glucose (mg/dl)
600
500
400
300
200
100
0
-30
0
30
60
90
120
Time after glucose dosing
Lean control
ob/ob control
YH18421 1 mg/kg
YH18421+Linagliptin (1+0.5) mg/kg
Linagliptin 0.5 mg/kg
B
AUCglucose(mg/dl)
600
500
400
300
200
100
0
***
Lean control
ob/ob control
YH18421
1 mg/kg
YH18421+Linagliptin
1+0.5 mg/kg
Linagliptin
0.5 mg/kg

Figure 23. Combined treatment of YH18421 and DPP-IV inhibitor improved acute glucose tolerance in *ob/ob* mice (n=8). *ob/ob* mice were treated with compounds or vehicle after 30 min glucose bolus (2 g/kg) and blood samples were collected over 2 hr. A, OGTT in *ob/ob* mice treated with vehicle (0.5% MC, closed circle), YH18421 (1 mg/kg, open square), YH18421+linagliptin (1+0.5 mg/kg, closed square) or linagliptin (0.5 mg/kg, open triangle) on the day 1. B, The area under the plasma glucose concentration–time curve for 2 h ($AUC_{0-2\ h}$) in an OGTT. Results are presented as the mean ± SEM. ***$P < 0.001$ versus vehicle.


A
Blood glucose (mg/dl)
600
500
400
300
200
100
0
-30
0
30
60
90
120
Time after glucose dosing
Lean control
ob/ob control
YH18421 1 mg/kg
YH18421+Linagliptin (1+0.5) mg/kg
Linagliptin 0.5 mg/kg

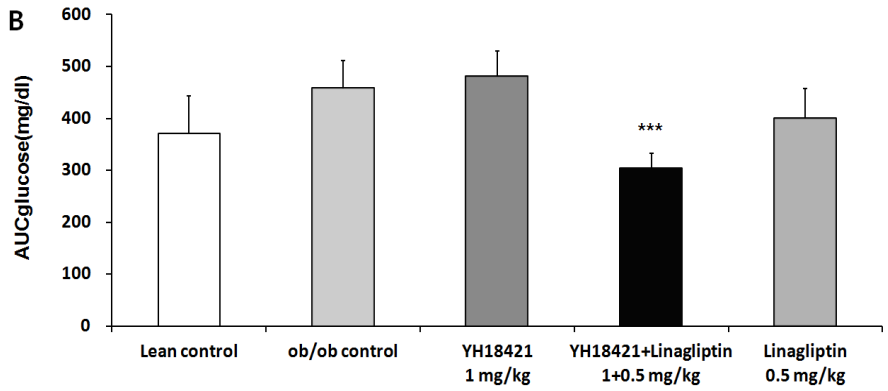

B
AUCglucose(mg/dl)
600
500
400
300
200
100
0
***
Lean control
ob/ob control
YH18421
1 mg/kg
YH18421+Linagliptin
1+0.5 mg/kg
Linagliptin
0.5 mg/kg

Figure 24. Repeated treatment of YH18421 and DPP-IV inhibitor improved chronic glucose tolerance in *ob/ob* mice (n=8). On the day 28 following daily dosing of YH18421 and linagliptin, *ob/ob* mice were treated with vehicle or compounds after 30 min glucose bolus (2 g/kg) and blood samples were collected over 2 hr. A, OGTT in *ob/ob* mice treated with vehicle (0.5% MC, closed circle), YH18421 (1 mg/kg, open square), YH18421+linagliptin (1+0.5 mg/kg, closed square) or linagliptin (0.5 mg/kg, open triangle) on the day 28. B, The area under the plasma glucose concentration–time curve for 2 h ($AUC_{0-2\ h}$) in an OGTT on the day 28. Results are presented as the mean ± SEM. ***P < 0.001 versus vehicle.

12. YH18421 has no effects on the muscle cell metabolism

GPR119 agonists were known to be generally well tolerated without notable adverse effects in animals and humans. One safety concern for GPR119 agonist was reported that focused on the effects on cardiac and skeletal muscle metabolism (Cornall et al. 2013; Cornall et al. 2015). In their report, GPR119 agonist (PSN632408) appeared to have harmful effects on cardiac and skeletal muscle cells by affecting cell viability and gene expression involved in fatty acid oxidation and insulin resistance. Thus effects of YH18421 on cell viability and the expression of AMPKα2, PPARα, PGC-1α and SOCS3 genes were examined in myotubes derived from C2C12 skeletal and H9c2 cardiac muscle cells. Metabolic functions of each gene are shown in Table 5. Contrary to results of PSN632408 in the report, there were no significant changes in cell viability of C2C12 and H9c2 cells treated with YH1421 (Figure 25). Furthermore, expression of AMPKα2, PPARα, PGC-1α and SOCS3 genes in C2C12 and H9c2 myotubes were unaffected by YH18421 treatment (Figure 26, 27). These results imply that possible detrimental effects of GPR119 activation on skeletal and cardiac muscle cells are compound-related, not GPR119 agonism

itself.

Table 5. Genes involved in skeletal and cardiac muscle metabolism

Gene	Main metabolic functions	References
AMPKα2	Fatty acid oxidation Mitochondrial biogenesis Muscle fiber type plasticity	Sriwijitkamol A et al. 2007
PPARα	Fatty acid oxidation Oxidative phosphorylation	Zhang J et al. 2004
PGC-1α	Mitochondrial biogenesis Fatty acid oxidation	Gastaldi G et al. 2007
SOCS3	Inhibits insulin signalling Insulin resistance	Jorgensen SB et al. 2013

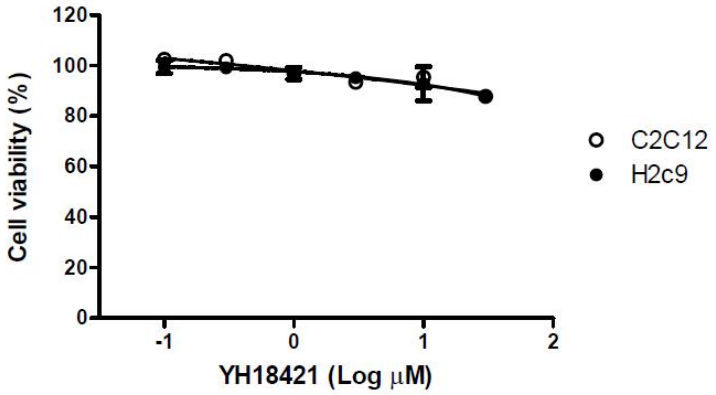


Figure 25. Effect of YH18421 on the viability of C2C12 and H2c9 cells. Cells were incubated with YH18421 for 2 days. Cell viability was measured with MTT colorimetric method. Triplicates for each concentration were used and the results are presented as the mean ± SEM.

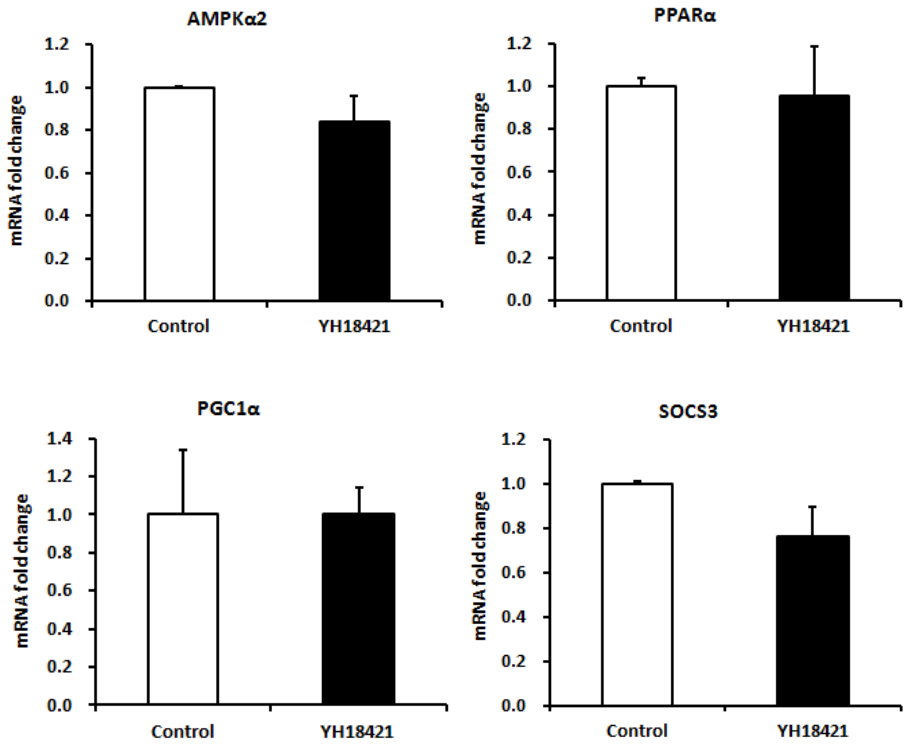


Figure 26. Effect of YH18421 on the gene expression in C2C12 myotubes. Differentiated C2C12 myotubes were treated with 100 nM YH18421 for 6 hr. mRNA abundance of each genes was normalized to GAPDH and represented as fold change relative to control. Triplicates for each sample were used and the results are presented as the mean ± SEM.

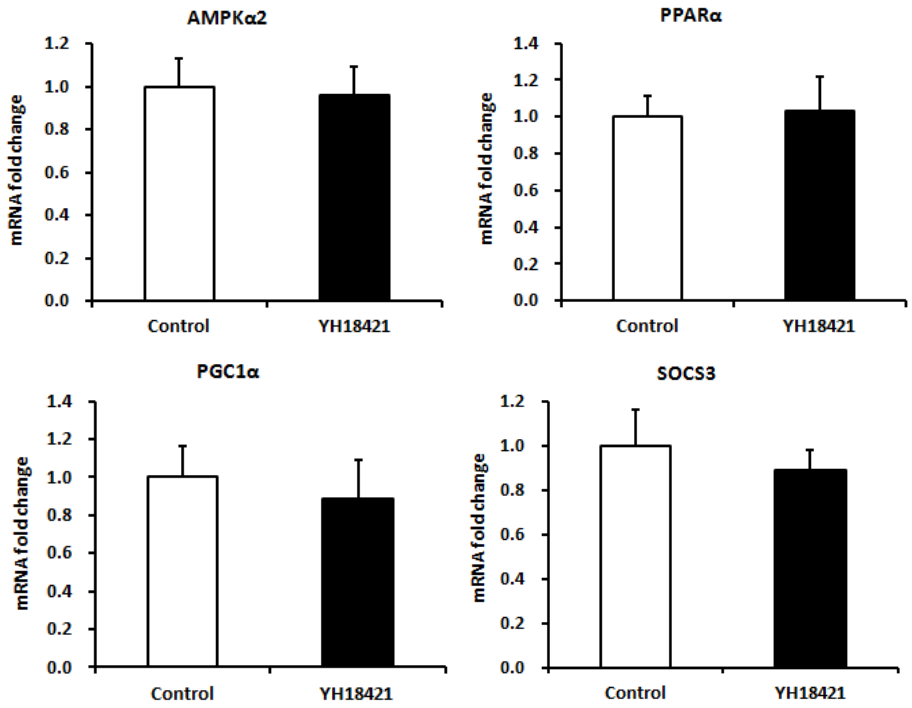


Figure 27. Effect of YH18421 on the gene expression in H9c2 myotubes. Differentiated H9c2 myotubes were treated with 100 nM YH18421 for 6 hr. mRNA abundance of each gene was normalized to GAPDH and represented as fold change relative to control. Triplicates for each sample were used and the results are presented as the mean ± SEM.

13. Pharmacokinetics of YH18421 in normal mice

Orally delivered pharmacologically active compounds must have favorable pharmacokinetic properties to provide adequate systemic exposure to elicit a pharmacodynamic response. In order to determine oral bioavailability of YH18421, pharmacokinetics with YH18421 in normal mice was performed. Plasma concentrations of YH18421 indicated that YH18421 was rapidly absorbed with the T_{max} of 0.5 hr. After reaching the maximum concentration, plasma concentration for YH18421 declined in a biphasic manner. C_{max} and $AUC_{0\text{-}24hr}$ for YH18421 increased as dose increased. Although the increase of C_{max} for YH18421 was less than dose proportional, the systemic exposure ($AUC_{0\text{-}24hr}$) for YH18421 increased in a dose proportional manner. Terminal half-lives for YH18421 were comparable across the dose levels with the values of 3.8 - 4.1 hr (Table 6). These pharmacokinetic properties suggest that YH18421 has excellent oral bioavailability and is suitable for oral administration.

Table 6. Pharmacokinetic parameters of YH18421 following oral administration in normal C57BL/6 mice (n=3/time point)

Pharmacokinetic parameters	YH18421	
	1 mg/kg	10 mg/kg
C_{max} (ng/mL)	102.4	503.4
T_{max} (hr)	0.5	0.5
$AUC_{0\text{-}24hr}$ (ng·hr/mL)	331.9	3028.1
AUC_{inf} (ng·hr/mL)	335.8	3067.1
$t_{1/2}$ (hr)	3.8	4.1

14. YH18421 has no influence on CYP3A4 induction

Polypharmacy, defined as the use of multiple pharmacological medications, is inevitable when treating patients with T2DM, given the large number of treatment options available. Moreover, concomitant metabolic diseases such as hypertension and dyslipidemia may require one or more drugs (May and Schindler 2016). An increasing number of concomitantly taken medications elevate the risk of the patient experiencing adverse drug effects from drug-drug interactions (Roger 2006). Members of cytochromes P450 (CYPs) constitute the major enzyme family capable of catalyzing the oxidative biotransformation of most drugs and are therefore of particular relevance for clinical drug-drug interactions (Nelson et al. 2004; Guengerich et al. 2008; Zanger et al. 2008). Members of the CYP3A subfamily are particularly important because CYP3A4 alone is responsible for the metabolism of 50-60% of all prescription drugs (Michalets 1998; Guengerich 1999). To investigate CYP3A4-mediated drug–drug interaction potential of YH18421, PXR (Pregnane X receptor)-based CYP 3A4 induction assay was developed using transient expression of PXR with a reporter that expresses PXR response element-luciferase. PXR is considered as the principal transcriptional regulator of CYP3A4 induction by xenobiotics (Savas et al. 1999; Waxman 1999). In this functional cell-based assays,

expression plasmids for the full length PXR are transfected with reporter plasmids that contain an intact promoter of a PXR target gene, CYP3A4 into hepG2 cells. The presence of a xenobiotic PXR ligand results in increased expression of the CYP3A4 reporter gene (Willson and Kliewer 2002) (Figure 28). Using this assay system, the effects of YH18421 on CYP3A4 induction potential were examined along with rifampicine, known CYP3A4 inducer. First, cytotoxic effect of YH18421 on hepG2 cells was examined. YH18421 didn't affect the viability of hepG2 cells (Figure 29). Then in a reporter gene assay, YH18421 showed no CYP3A4 induction potential upto 30 μM (maximum soluble concentration in media) whereas rifampicine effectively induced PXR-induced CYP3A4 expression (Figure 30). These results indicate that drug-drug interaction potential of YH18421 can be minimized.

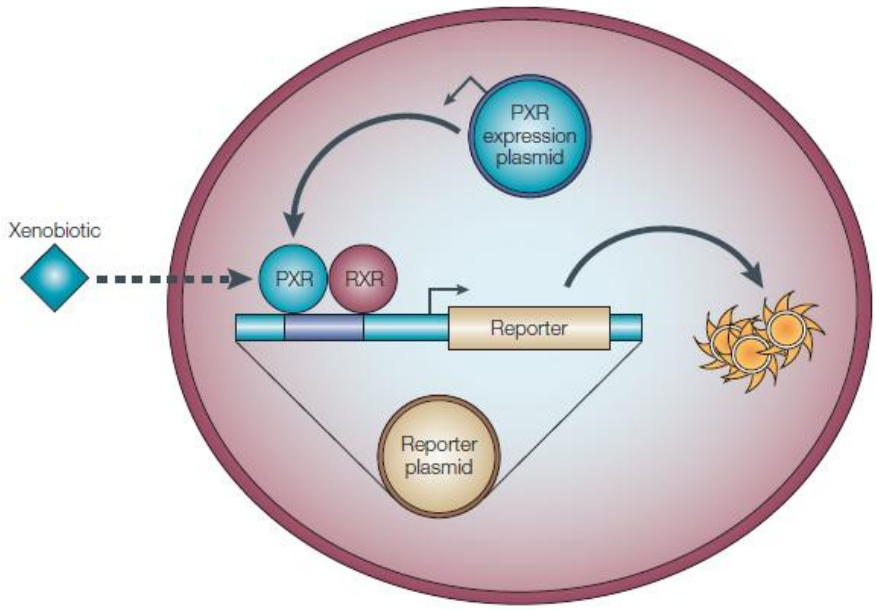


Figure 28. PXR based CYP3A4 induction. HepG2 cell is transfected with an expression plasmid for PXR and a reporter plasmid. As a result, the PXR is expressed within the cells, where it dimerizes with endogenous RXR. The PXR–RXR heterodimer binds to CYP3A4 promoter sequences in the reporter plasmid. The presence of a xenobiotic PXR ligand results in increased expression of the reporter gene **(**Willson and Kliewer 2002).

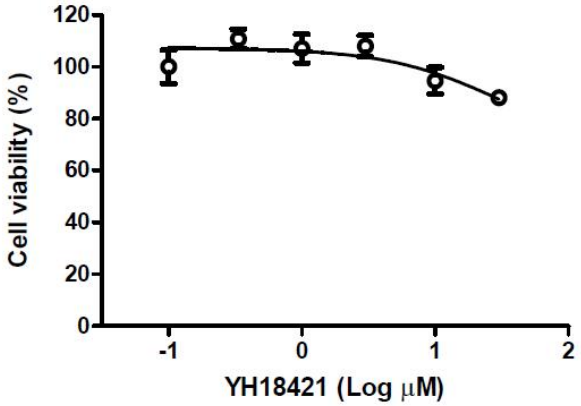


Figure 29. Effect of YH18421 on the viability of hepG2 cells. Cells were incubated with YH18421 for 2 days. Cell viability was measured with MTT colorimetric method. Triplicates for each concentration were used and the results are presented as the mean ± SEM.

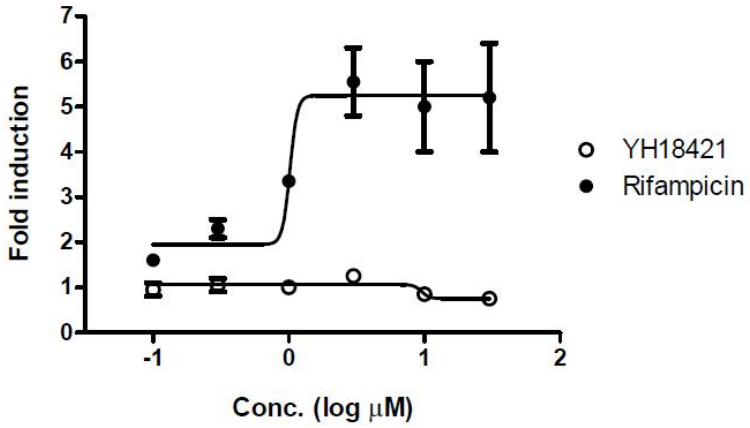


Figure 30. Effect of YH18421 on CYP3A4 induction. HepG2 cells were transiently expressing PXR and reporter genes were incubated with YH18421 and rifampicine for 24 hr. CYP3A4 induction was measured with PXRE-luciferase reporter gene. Triplicates for each concentration were used and the data are represented as fold induction relative to control.

Discussion

Although a number of pharmacological agents including metformin, sulfonylurea, and thiazolidinedione are available for the treatment of T2DM, less than one-third patients are reported to achieve desired levels of glycemic control (Koro et al. 2004). Furthermore, responsiveness to these currently available therapies declines during long-term treatment and the side effects including hypoglycemia, weight-gain, bone loss and gastrointestinal problems limit their long-term use (UKPDS 1998). Therefore, new pharmacological treatments for T2DM with novel mechanisms of action are needed.

Steady progress has been made toward the development of orally-active small molecule GPR119 agonists for effective T2DM treatment (Jones et al. 2009). However, a specific agonist with high potency and sustainable glycemic control has been proved to be elusive.

It has been reported that oleolyethanolamide (OEA), an endogenous GPR119 ligand, enhances cAMP accumulation in GPR119-transfected HEK293 cells with an EC_{50} value of 3.2 uM (Overton et al. 2006). YH18421 has an EC_{50} value of 2.2 nM for human

GPR119 activation which is approximately 1000-fold more potent than OEA. Also, potency of YH18421 was compared with that of clinical GPR119 agonists and it was found that YH18421 is approximately 20 fold more potent than clinical candidates. Importantly, YH18421 was inactive towards other off target GPCRs, demonstrating the potency and specificity for GPR119 agonism.

Increased cAMP accumulation by YH18421 resulted in insulin release in the HIT-T15 insulinoma cells and GLP-1 release in the GLUTag L cells, both of which express GPR119 endogenously (Lauffer et al. 2009; Chu et al. 2010). Single oral administration of YH18421 in normal mice effectively reduced blood glucose during OGTT and increased plasma GLP-1 and insulin. These *in vitro* secretion and *in vivo* pharmacodynamic results are consistent with reports that GPR119 agonist plays a dual role in glycemic control by enhancing both GLP-1 and insulin secretion (Chu et al. 2007; Chu et al. 2008).

DPP-IV inhibitors are widely used as oral GLP-1 based therapy for T2DM. A major advantage of the DPP-IV inhibitors is their weight neutrality, with no associated risk of hypoglycemia observed in clinical studies. However, a major drawback of DPP-IV inhibitors is their weaker potency, with a modest HbA1c reduction of 0.5-1% in T2DM

patients in clinical trials (Michael et al. 2007). In agreement with this report, DPP-IV inhibitors evidently do not raise plasma active GLP-1 levels sufficiently enough to induce strong efficacy and body weight reduction (Drucker et al. 2006). Thus, GLP-1 releasing GPR119 agonist could synergistically cooperate with DPP-IV inhibitor which prevents GLP-1 degradation.

In this study, two representative marketed DPP-IV inhibitors (linagliptin and sitagliptin) were synthesized and combined with YH18421. YH18421 acted synergistically with a DPP-IV inhibitor in increasing plasma active GLP-1 levels and this combination led to additive blood glucose lowering during mouse OGTT. Based on this finding, I reason that in combination with a DPP-IV inhibitor, YH18421 could potentially maximize active blood GLP-1 levels and mitigate drawbacks associated with DPP-IV inhibitors.

The diet-induced obese (DIO) mouse model offers human-like, translational relevance, in which the pathogenesis of diabetes and obesity is based on natural factors, including excessive calorie intake (Wang and Liao 2012), and has been used for the study of GPR119 agonists (Ha et al. 2014; Al-Barazanji et al. 2015). After repeated administration of YH18421 in DIO mice for 4 weeks, improved glucose

tolerance by YH18421 lasted until day 28 suggesting that the efficacy of YH18421 is sustained during the treatment period.

Several early GPR119 agonists have entered into clinical studies, however, tachyphylaxis (a decline in efficacy after repeated dosing) has been observed for some GPR119 agonists such as GSK1292263 and JNJ-38431055 (Nilsson et al. 2012; Katz et al. 2012). Single-dose administration of these agonists decreased glucose excursion during an OGTT, but multiple-dose administration for 2 weeks did not reduce 24-h weighted mean glucose levels. In an interesting report by researchers at Array Biophrama, a clinical GPR119 agonist (clinical compound 1) which showed tachyphylaxis in a clinical study was evaluated in a high fat diet ZDF (Zucker diabetic fatty) rat model for 4 weeks (Jay 2012). Clinical compound 1 reduced glucose excursion after an OGTT on the day 1, but had no effect on glucose excursion or fasted glucose after 28 days. I assume clinical compound 1 to be GSK1292263 or JNJ-38431055 based on the description in the literature (Katz et al. 2012; Jay 2012; Nilsson et al. 2012), and these findings imply that chronic preclinical studies using rodent diabetes models can predict tachyphylaxis in clinical studies. I therefore expect that YH18421 would have sustained glucose lowering effects without tachyphylaxis in

clinical settings, based on the results from our chronic DIO mouse model study.

One of the major unmet medical needs for current diabetes therapies is weight gain, which has been associated with some approved drugs including sulphonylureas, thiazolidinediones and insulin injections, and there remains a critical need for anti-diabetic drugs effective for both weight and glycemic control (American Diabetes Association 2014). YH18421 was observed to suppress weight gain at a dose level higher than that needed to improve glycemic control during chronic treatment in DIO mice. In general, a reduction in weight has been observed at doses significantly higher than those required to impact glucose homeostasis (Chu et al. 2007). Reduced food intake by increased GLP-1 and PYY levels by YH18421 is a plausible explanation for the mechanism of YH18421 in causing weight loss in DIO mouse.

Regarding safety aspects of GPR119 agonist, clinical studies have shown that GPR119 agonists were safe and generally well tolerated (Katz et al. 2012; Nunez et al. 2014). One safety concern for GPR119 agonist was reported that focused on the effects of GPR119 on cardiac and skeletal muscle (Cornall et al. 2013; Cornall et al. 2015). In their

studies, GPR119 activation appeared to have detrimental effects on cardiac and skeletal muscle cells *in vitro*, although *in vivo* effects were not determined. We couldn't find any negative effects of YH18421 on cells derived from cardiac and skeletal muscle. Also, in our 2-week repeated dose mouse toxicity studies with YH18421, no pathological abnormalities on cardiac and skeletal muscle tissues or drug-related systemic toxic signs were found.

In terms of the DMPK (drug metabolism and pharmacokinetics) aspects of YH18421, results from mouse pharmacokinetics and cell based CYP3A4 induction assay indicate that YH18421 is optimal for oral administration and has low potential of drug-drug interaction.

Besides the role of GPR119 in the control of diabetes and obesity, recent research demonstrated that GPR119 receptor was expressed in hepatocytes and clinical GPR119 agonists (MBX2982 and APD668) reduced hepatic steatosis in high-fat diet induced mouse model of non-alcoholic steatohepatitis (NASH) (Yang et al. 2016; Bahirat et al. 2017). Even though the prevalence of NASH is rapidly growing, there are currently no FDA-approved drug therapies for the disease (Younossi et al. 2016). These reports suggest the potential benefit of GPR119 agonist for the treatment of NASH. In addition, in a clinical trial with

GSK1292263, a GPR119 agonist lowered fasting LDL-cholesterol and triglycerides, while increasing HDL-cholesterol in diabetic and non-diabetic dyslipidemic patients, suggesting that GPR119 agonist also acts as an anti-dyslipidemic agent (Nunez et al. 2012). Thus, these data suggest that GPR119 agonist may be useful for the treatment of NASH and dyslipidemia as well as T2DM. Further studies are required to delineate the role of YH18421 in the regulation of NASH and dyslipidemia. In summary, a novel small-molecule GPR119 agonist, YH18421 was identified which has shown sustainable blood glucose control with the additional benefit of weight loss in diabetic animal models. It was also observed that combination of YH18421 with a DPP-IV inhibitor was effective in maximizing active plasma GLP-1 levels and potentiating glycemic control. Therefore I suggest that YH18421, alone or in combination with a DPP-IV inhibitor, represents a new type of anti-diabetic agent as an oral GLP-1 based therapy for the treatment of T2DM.

Conclusion

The present study identified an orally available novel small-molecule GPR119 agonist, YH18421 and validated its potential to elicit glycemic control in mouse models of diabetes. YH18421 improved glucose tolerance in normal mouse with increased blood GLP-1 and insulin and has shown sustainable blood glucose control with the additional benefit of weight loss in chronic diabetic mouse model. The high potency *in vitro* and *in vivo* durable efficacy could help YH18421 overcome the limitations of current clinical GPR119 agonists such as weak efficacy or tachyphylaxis. Combination treatment of YH18421 with a DPP-IV inhibitor was effective in maximizing active plasma GLP-1 levels and potentiating glycemic control.

In conclusion, by enhancing GSIS and active GLP-1 release, YH18421 is expected to exert improved glucose control and body weight loss leading to amelioration of diabetes (Figure 31). The results of this study suggest that YH18421, alone or in combination with a DPP-IV inhibitor, represents a new type of an oral GLP-1 based therapy for the treatment of T2DM.

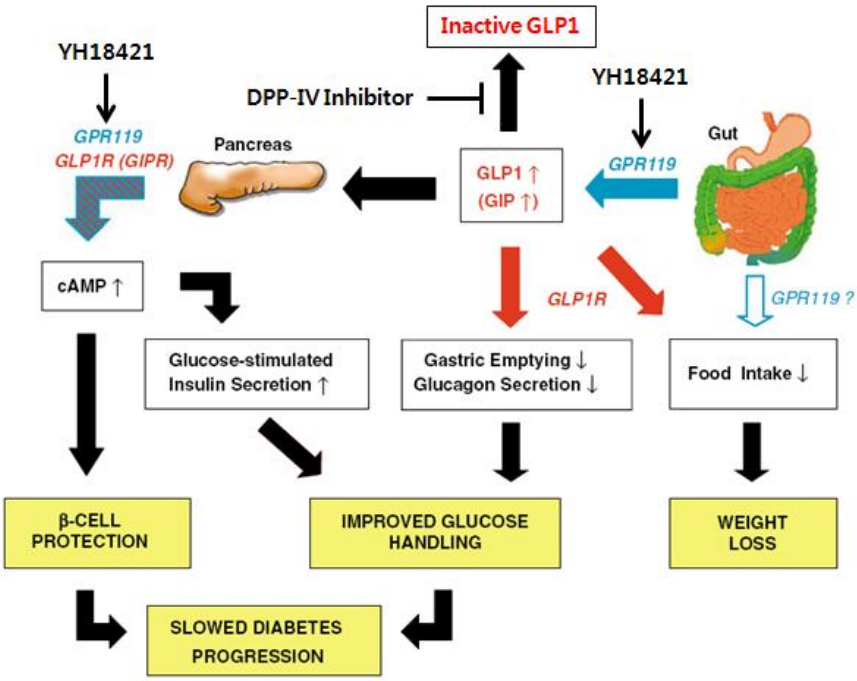


Figure 31. Schematic diagram showing possible anti-diabetic actions of YH18421 combined with DPP-IV inhibitor (Modified from Overton et al. 2008)

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국문 초록

G 단백질 결합 수용체 119 (GPR119)는 소장 L 세포에서 GLP-1을 분비 시키는 동시에, 췌장 β 세포에서 glucose 의존적 인슐린 분비 (GSIS)를 촉진시키는 이중 작용으로 인해 제 2 형 당뇨병 치료제 개발을 위한 유망한 표적으로 알려졌다. 이러한 GPR119의 이중 작용 기전의 이해와 더불어 소분자 GPR119 agonist의 개발은 제 2 형 당뇨병 치료제로서 많은 관심을 불러 일으켰다. 특히 GLP-1의 분해를 차단하는 DPP-4 억제제와 병용투여 함으로써 활성형 GLP-1의 상승작용을 일으킬 수 있다. 본 연구에서는 새로운 화학구조의 GPR119 agonist인 YH18421을 동정하고, 당뇨병 치료제로서의 가능성을 평가하였다. YH18421은 in vitro 세포 기반 시스템에서 인간 GPR119를 특이적으로 활성화 시켰으며, GLP-1 및 glucose 의존적 인슐린 분비 (GSIS)를 촉진하였다. In vivo 실험으로 정상 생쥐에서 YH18421의 단일 경구 투여에 의해 내당능이 향상되었다. 추가로 YH18421과 DPP-4 억제제 병용 투여의

상승작용으로 인해 혈중 활성 형 GLP-1 농도가 증폭되었으며, 이는 YH18421 또는 DPP-4 억제제 단독 투여에 비해 추가적인 혈당 강하 효과를 일으켰다. 식이 유도 비만(DIO) 마우스 당뇨병 모델에서 YH18421 4 주 반복 투여 후 혈당강하 효과가 유지되었고, 마찬가지로 DPP-IV 억제제와 병용 투여 했을 때, 추가적인 혈당 강하 효과가 관찰되었다. 또한 DIO 마우스 비만 모델에서 YH18421의 4 주간 반복투여 후, 체중 증가의 억제 현상을 관찰하였다. 이러한 결과들로 미루어 볼 때, YH18421은 당뇨병 상태에서 지속적인 혈당 조절 및 체중 증가 억제 효과를 제공하며, 나아가 YH18421 단독 또는 DPP-IV 억제제와의 병용투여는 제 2 형 당뇨병 치료에 효과적인 경구용 GLP-1 기반 치료법일 수 있음을 시사한다.

Keywords : **YH18421/ GPR119/ DPP-IV inhibitor/ GLP-1/ Insulin/ Type 2 diabetes**